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(54) Title: PRETARGETING METHODS AND COMPOUNDS

(57) Abstract

Methods, compounds, compositions and kits that relate to pretargeted delivery of diagnostic and therapeutic agents are disclosed. In particular, methods and agents for reducing the immunogenicity of targeting moiety-anti-ligand conjugates or other components employed in diagnostic and therapeutic pretargeting protocols.

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PRETARGETING METHODS AND COMPOUNDS

Technical Field

The present invention relates to methods, compounds, compositions and kits useful for delivering to a target site a targeting moiety that is conjugated to one member of a ligand/anti-ligand pair. After localization and clearance of the targeting moiety conjugate, direct or indirect binding of a diagnostic or therapeutic agent conjugate at the target site occurs. Methods for reducing the immunogenicity of immunogenic components administered in conducting such diagnostic or therapeutic protocols are discussed.

Background of the Invention

Conventional cancer therapy is plagued by two problems. The generally attainable targeting ratio (ratio of administered dose localizing to tumor versus administered dose circulating in blood or ratio of administered dose localizing to tumor versus administered dose migrating to bone marrow) is low. Also, the absolute dose of radiation or therapeutic agent delivered to the tumor is insufficient in many cases to elicit a significant tumor response. Improvement in targeting ratio or absolute dose to tumor is sought.

Summary of the Invention

The present invention is directed to diagnostic and therapeutic pretargeting methods, moieties useful therein and methods of making those moieties. Such pretargeting methods are characterized by an improved targeting ratio or increased absolute dose to the target cell sites in comparison to conventional cancer therapy.

The present invention provides targeting moiety-anti-ligand, such as avidin or streptavidin, compounds useful in diagnostic and therapeutic pretargeting methods. Preparation and purification of such anti-ligand-targeting moiety compounds are also discussed. Reduction of the immunogenicity of this targeting moiety-anti-ligand component is also provided in accordance with the present invention (two-step pretargeting). Reducing the immunogenicity of targeting moiety-ligand conjugate is at issue for three-step pretargeting.

Immunogenicity reduction may be accomplished for conjugates having an antibody component by employing antibodies or fragments thereof exhibiting human character (e.g., chimeric, human or humanized antibodies). Chimeric antibodies and, more preferably, human or humanized antibodies exhibit lower immunogenicity when administered to humans than antibodies produced in or from other mammalian species.

In addition, targeting moieties of lower molecular weight are generally less immunogenic than their higher molecular weight counterparts. For example, antibody fragments are generally less immunogenic than whole antibodies as are small molecule targeting moieties.

Similarly, the targeting moiety-anti-ligand conjugate may be chemically altered to decrease the immunogenicity thereof. Either or both the targeting

moiety or the anti-ligand portion of the conjugate may be so altered; provided that the targeting moiety retains its targeting ability and the anti-ligand retains its capability to bind ligand with high affinity. For example, the conjugate or any component thereof may be chemically modified via polyethyleneglycol (PEG) derivatization (i.e., PEGylation) or via polymer derivatization (e.g., using poly-amino acids, vinyl polymers, dextrans or the like). Chemical modification in accordance with the present invention includes charge modification, such as modification via succinyl derivatization (i.e, succinylation).

Immunogenicity-reducing modification of the tertiary structure of a proteinaceous immunogen may also be employed in the practice of the present invention. Such tertiary structure modification is preferably conducted by recombinant techniques to reduce the number or immunogenicity of immunogenic sites on the proteinaceous immunogen.

Alternatively, immunosuppressive agents may be administered prior to, concurrently with, or following administration of the targeting moiety-anti-ligand conjugate. Preferred immunosuppressive agents include cyclosporin A, verapamil, mycophenolic acid, transforming growth factor-beta, deoxyspergualin, FK506, rapamycin, azathioprine cyclophosphamide, immunophilins such as FK binding protein 12, peptide derivatives of non-erythroid spectin, and combinations thereof. Liposome encapsulated or particulate immunosuppressive agents may also be employed.

Clearing agents of the present invention which rapidly remove circulating targeting moiety-anti-ligand conjugate have also shown utility as immunosuppressive agents in the practice of the methods of the present invention. Extracorporeal, particulate and other clearing agents and mechanisms

are also contemplated for use within the present invention.

Local administration of immunogenic therapeutic or diagnostic agents may be employed to reduce the immunogenicity of such components.

Non-specific pretargeting techniques are also contemplated by the present invention. Such pretargeting techniques contemplate administration of a non-specific targeting moiety-ligand or a non-specific targeting moiety-anti-ligand conjugate. The non-specific targeting moiety becomes trapped physically or kinetically in the extravascular compartment and is cleared from the vascular compartment by the recipient's excretory mechanisms or via the use of a clearing agent or clearing mechanism in accordance with the present invention. Preferably, the non-specific targeting moiety-containing conjugate is administered, equilibrates between the extravascular compartment and the vascular compartment and the conjugate in the vascular space is cleared using a clearing agent or a clearing mechanism. Active agent-anti-ligand or active agent-ligand conjugate is then administered and such active agent-containing conjugate accretes to the extravascular compartment localized non-specific targeting moiety-containing conjugate. Non-specific pretargeting allows the use of large, non-immunogenic proteins (e.g., IgG and IgM) for radioimmunotherapy, for example. Irrelevant antibodies and other non-specific targeting moieties may also be used in accordance with this aspect of the present invention. Short half-life active agents, such as alpha-emitters, exert a therapeutic effect within a short time frame and are therefore particularly amenable to non-specific pretargeting.

The present invention also provides an article of manufacture which includes packaging material and a

first conjugate contained within the packaging material, wherein the first conjugate incorporates a targeting moiety and streptavidin, and wherein the first conjugate is capable of localizing at a target site upon administration to a mammalian recipient, and streptavidin retains the ability to bind to biotin, and further wherein the packaging material includes a label, which label identifies the targeting moiety component of the first conjugate, identifies the streptavidin component of the first conjugate and indicates an appropriate use of the first conjugate in human recipients.

Brief Description of the Drawings

Figure 1 illustrates blood clearance of biotinylated antibody following intravenous administration of avidin.

Figure 2 depicts radiorhenium tumor uptake in a three-step pretargeting protocol, as compared to administration of radiolabeled antibody (conventional means involving antibody that is covalently linked to chelated radiorhenium).

Figure 3 depicts the tumor uptake profile of NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody.

Figure 4 depicts the tumor uptake and blood clearance profiles of NR-LU-10-streptavidin conjugate.

Figure 5 depicts the rapid clearance from the blood of asialoorosomucoid in comparison with orosomucoid in terms of percent injected dose of I-125-labeled protein.

Figure 6 depicts the 5 minute limited biodistribution of asialoorosomucoid in comparison with orosomucoid in terms of percent injected dose of I-125-labeled protein.

Figure 7 depicts NR-LU-10-streptavidin conjugate blood clearance upon administration of three controls (○, ●, ■) and two doses of a clearing agent (⌘, □) at 25 hours post-conjugate administration.

Figure 8 shows limited biodistribution data for LU-10-StrAv conjugate upon administration of three controls (Groups 1, 2 and 5) and two doses of clearing agent (Groups 3 and 4) at two hours post-clearing agent administration.

Figure 9 depicts NR-LU-10-streptavidin conjugate serum biotin binding capability at 2 hours post-clearing agent administration.

Figure 10 depicts NR-LU-10-streptavidin conjugate blood clearance over time upon administration of a control (○) and three doses of a clearing agent (▽, △, □) at 24 hours post-conjugate administration.

Figure 11A depicts the blood clearance of LU-10-StrAv conjugate upon administration of a control (PBS) and three doses (50, 20 and 10 μg) of clearing agent at two hours post-clearing agent administration.

Figure 11B depicts LU-10-StrAv conjugate serum biotin binding capability upon administration of a control (PBS) and three doses (50, 20 and 10 μg) of clearing agent at two hours post-clearing agent administration.

Figure 12 depicts the prolonged tumor retention of NR-LU-10-streptavidin conjugate (▲) relative to NR-LU-10 whole antibody (△) over time.

Figure 13 depicts the prolonged liver retention of a pre-formed complex of NR-LU-10-biotin (○ ; chloramine T labeled with I-125) complexed with streptavidin (● ; PIP-I-131 labeled).

Figure 14 depicts the prolonged liver retention of Biotin-PIP-I-131 label relative to the streptavidin-NR-LU-10-(PIP-I-125) label.

Figure 15A depicts tumor uptake for increasing doses of PIP-Biocytin in terms of %ID/G.

Figure 15B depicts tumor uptake for increasing doses of PIP-Biocytin over time in terms of pMOL/G.

Figure 16A depicts tumor versus blood localization of a 0.5 μg dose of PIP-Biocytin over time in terms of %ID/G.

Figure 16B depicts tumor versus blood localization of a 0.5 μg dose of PIP-Biocytin in terms of %ID.

Figure 17A depicts tumor uptake of LU-10-StrAv and PIP-Biocytin over time in terms of %ID/G.

Figure 17B depicts blood clearance of LU-10-StrAv and PIP-Biocytin over time in terms of %ID/G.

Figure 18 depicts PIP-Biocytin:LU-10-StrAv ratio in tumor and blood over time.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

Targeting moiety: A molecule that binds to a defined population of cells. The targeting moiety may bind a receptor, an oligonucleotide, an enzymatic substrate, an antigenic determinant, or other binding site present on or in the target cell population. Antibody is used throughout the specification as a prototypical example of a targeting moiety. Tumor is used as a prototypical example of a target in describing the present invention.

Non-specific targeting moiety: A molecule that binds to a variety of cells including target cells of diagnostic or therapeutic protocols as well as non-target cells. Preferably, the target cells are located within a separate physiological compartment than non-target cells (e.g., the target cells are located in the extravascular space while non-target cells are not). Non-specific targeting moieties may be antibody-based, peptide-based, polymer-based, microparticulate-based, aggregate-based (e.g.,

aggregated streptavidin, avidin or the like) or the like.

Ligand/anti-ligand pair: A complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include zinc finger protein/dsDNA fragment, enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, and biotin/avidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

Anti-ligand: As defined herein, an "anti-ligand" demonstrates high affinity, and preferably, multivalent binding of the complementary ligand. Preferably, the anti-ligand is large enough to avoid rapid renal clearance, and contains sufficient multivalency to accomplish crosslinking and aggregation of targeting moiety-ligand conjugates. Univalent anti-ligands are also contemplated by the present invention. Anti-ligands of the present invention may exhibit or be derivatized to exhibit structural features that direct the uptake thereof, e.g., galactose residues that direct liver uptake. Avidin and streptavidin are used herein as prototypical anti-ligands.

Avidin: As defined herein, "avidin" includes avidin, streptavidin and derivatives and analogs thereof that are capable of high affinity, multivalent or univalent binding of biotin.

Ligand: As defined herein, a "ligand" is a relatively small, soluble molecule that exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human. Biotin is used as the prototypical ligand.

Active Agent: A diagnostic or therapeutic agent ("the payload"), including radionuclides, drugs, anti-tumor agents, toxins and the like. Radionuclide

therapeutic agents are used as prototypical active agents.

N$_x$S$_y$ Chelates: As defined herein, the term "N$_x$S$_y$ chelates" includes buoy chelators that are capable of (i) coordinately binding a metal or radiometal and (ii) covalently attaching to a targeting moiety, ligand or anti-ligand. Particularly preferred N$_x$S$_y$ chelates have N_2S_2 and N_3S cores. Exemplary N$_x$S$_y$ chelates are described in Fritzberg et al., Proc. Natl. Acad. Sci. USA 85:4024-29, 1988; in Weber et al., Bioconj. Chem. 1:431-37, 1990; and in the references cited therein, for instance.

Pretargeting: As defined herein, pretargeting involves target site localization of a targeting moiety that is conjugated with one member of a ligand/anti-ligand pair; after a time period sufficient for optimal target-to-non-target accumulation of this targeting moiety conjugate, active agent conjugated to the opposite member of the ligand/anti-ligand pair is administered and is bound (directly or indirectly) to the targeting moiety conjugate at the target site (two-step pretargeting). Three-step and other related methods described herein are also encompassed.

Clearing Agent: An agent capable of binding, complexing or otherwise associating with an administered moiety (e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone) present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in circulation. The clearing agent is preferably characterized by physical properties, such as size, charge, configuration or a combination thereof, that limit clearing agent access to the population of target cells recognized by a

targeting moiety used in the same treatment protocol as the clearing agent.

Target Cell Retention: The amount of time that a radionuclide or other therapeutic agent remains at the target cell surface or within the target cell. Catabolism of conjugates or molecules containing such therapeutic agents appears to be primarily responsible for the loss of target cell retention.

Conjugate: A conjugate encompasses chemical conjugates (covalently or non-covalently bound), fusion proteins and the like.

Immunogenicity: A measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or cellular) when administered to a recipient. The present invention is concerned with the immunogenicity of the conjugates and their component parts.

A recognized disadvantage associated with in vivo administration of targeting moiety-radioisotopic conjugates for imaging or therapy is localization of the attached radioactive agent at both non-target and target sites. Until the administered radiolabeled conjugate clears from the circulation, normal organs and tissues are transitorily exposed to the attached radioactive agent. For instance, radiolabeled whole antibodies that are administered in vivo exhibit relatively slow blood clearance; maximum target site localization generally occurs 1-3 days post-administration. Generally, the longer the clearance time of the conjugate from the circulation, the greater the radioexposure of non-target organs.

These characteristics are particularly problematic with human radioimmunotherapy. In human clinical trials, the long circulating half-life of radioisotope bound to whole antibody causes relatively large doses of radiation to be delivered to the whole body. In

particular, the bone marrow, which is very radiosensitive, is the dose-limiting organ of non-specific toxicity.

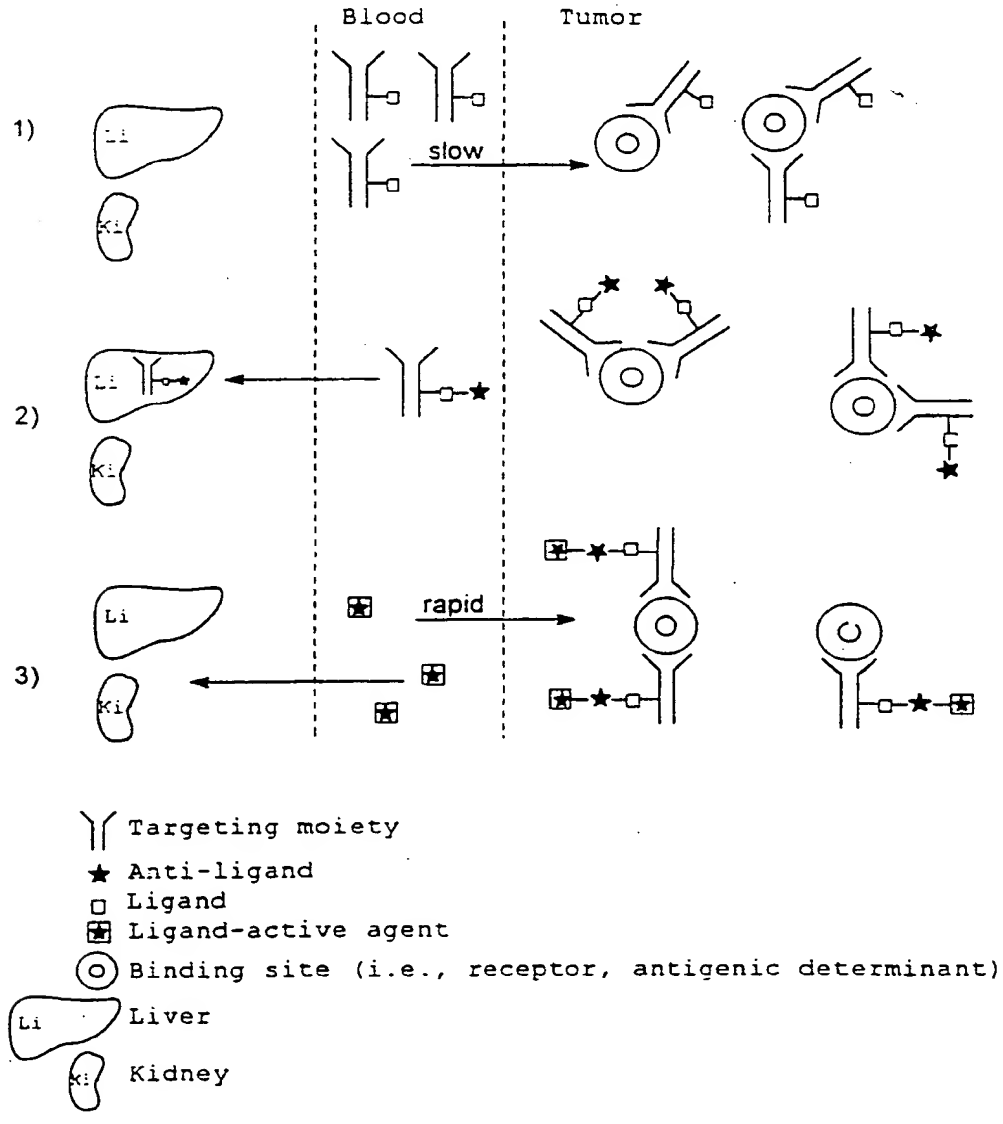
In order to decrease radioisotope exposure of non-target tissue, potential targeting moieties generally have been screened to identify those that display minimal non-target reactivity, while retaining target specificity and reactivity. By reducing non-target exposure (and adverse non-target localization and/or toxicity), increased doses of a radiotherapeutic conjugate may be administered; moreover, decreased non-target accumulation of a radiodiagnostic conjugate leads to improved contrast between background and target.

Therapeutic drugs, administered alone or as targeted conjugates, are accompanied by similar disadvantages. Again, the goal is administration of the highest possible concentration of drug (to maximize exposure of target tissue), while remaining below the threshold of unacceptable normal organ toxicity (due to non-target tissue exposure). Unlike radioisotopes, however, therapeutic drugs need to be taken into a target cell to exert a cytotoxic effect. In the case of targeting moiety-therapeutic drug conjugates, it would be advantageous to combine the relative target specificity of a targeting moiety with a means for enhanced target cell internalization of the targeting moiety-drug conjugate.

In contrast, enhanced target cell internalization is disadvantageous if one administers diagnostic agent-targeting moiety conjugates. Internalization of diagnostic conjugates results in cellular catabolism and degradation of the conjugate. Upon degradation, small adducts of the diagnostic agent or the diagnostic agent per se may be released from the cell, thus eliminating the ability to detect the conjugate in a target-specific manner.

One method for reducing non-target tissue exposure to a diagnostic or therapeutic agent involves "pretargeting" the targeting moiety at a target site, and then subsequently administering a rapidly clearing diagnostic or therapeutic agent conjugate that is capable of binding to the "pretargeted" targeting moiety at the target site. A description of some embodiments of the pretargeting technique may be found in US Patent No. 4,863,713 (Goodwin et al.).

A typical pretargeting approach ("three-step") is schematically depicted below.



Briefly, this three-step pretargeting protocol features administration of an antibody-ligand conjugate, which is allowed to localize at a target site and to dilute in the circulation. Subsequently administered anti-ligand binds to the antibody-ligand conjugate and clears unbound antibody-ligand conjugate from the blood. Preferred anti-ligands are large and contain sufficient multivalency to accomplish crosslinking and aggregation of circulating antibody-ligand conjugates. The clearing by anti-ligand is probably attributable to anti-ligand crosslinking and/or aggregation of antibody-ligand conjugates that are circulating in the blood, which leads to complex/aggregate clearance by the recipient's RES (reticuloendothelial system). Anti-ligand clearance of this type is preferably accomplished with a multivalent molecule; however, a univalent molecule of sufficient size to be cleared by the RES on its own could also be employed. Alternatively, receptor-based clearance mechanisms, e.g., Ashwell receptor or hexose residue, such as galactose or mannose residue, recognition mechanisms, may be responsible for anti-ligand clearance via the liver. Such clearance mechanisms are less dependent upon the valency of the anti-ligand with respect to the ligand than the RES complex/aggregate clearance mechanisms. It is preferred that the ligand-anti-ligand pair displays relatively high affinity binding.

A diagnostic or therapeutic agent-ligand conjugate that exhibits rapid whole body clearance is then administered. When the circulation brings the active agent-ligand conjugate in proximity to the target cell-bound antibody-ligand-anti-ligand complex, anti-ligand binds the circulating active agent-ligand conjugate and produces an antibody-ligand : anti-ligand : ligand-active agent "sandwich" at the target site. Because the diagnostic or therapeutic agent is

attached to a rapidly clearing ligand (rather than antibody, antibody fragment or other slowly clearing targeting moiety), this technique promises decreased non-target exposure to the active agent.

Alternate pretargeting methods eliminate the step of parenterally administering an anti-ligand clearing agent. These "two-step" procedures feature targeting moiety-ligand or targeting moiety-anti-ligand administration, followed by administration of active agent conjugated to the opposite member of the ligand-anti-ligand pair. As an optional step "1.5" in the two-step pretargeting methods of the present invention, a clearing agent (preferably other than ligand or anti-ligand alone) is administered to facilitate the clearance of circulating targeting moiety-containing conjugate.

In the two-step pretargeting approach, the clearing agent preferably does not become bound to the target cell population, either directly or through the previously administered and target cell bound targeting moiety-anti-ligand or targeting moiety-ligand conjugate. An example of two-step pretargeting involves the use of biotinylated human transferrin as a clearing agent for avidin-targeting moiety conjugate, wherein the size of the clearing agent results in liver clearance of transferrin-biotin-circulating avidin-targeting moiety complexes and substantially precludes association with the avidin-targeting moiety conjugates bound at target cell sites. (See, Goodwin, D.A., Antibod. Immunoconj. Radiopharm., 4: 427-34, 1991).

The two-step pretargeting approach overcomes certain disadvantages associated with the use of a clearing agent in a three-step pretargeted protocol. More specifically, data obtained in animal models demonstrate that in vivo anti-ligand binding to a pretargeted targeting moiety-ligand conjugate (i.e.,

the cell-bound conjugate) removes the targeting moiety-ligand conjugate from the target cell. One explanation for the observed phenomenon is that the multivalent anti-ligand crosslinks targeting moiety-ligand conjugates on the cell surface, thereby initiating or facilitating internalization of the resultant complex. The apparent loss of targeting moiety-ligand from the cell might result from internal degradation of the conjugate and/or release of active agent from the conjugate (either at the cell surface or intracellularly). An alternative explanation for the observed phenomenon is that permeability changes in the target cell's membrane allow increased passive diffusion of any molecule into the target cell. Also, some loss of targeting moiety-ligand may result from alteration in the affinity by subsequent binding of another moiety to the targeting moiety-ligand, e.g., anti-idiotype monoclonal antibody binding causes removal of tumor bound monoclonal antibody.

The present invention recognizes that this phenomenon (apparent loss of the targeting moiety-ligand from the target cell) may be used to advantage with regard to in vivo delivery of therapeutic agents generally, or to drug delivery in particular. For instance, a targeting moiety may be covalently linked to both ligand and therapeutic agent and administered to a recipient. Subsequent administration of anti-ligand crosslinks targeting moiety-ligand-therapeutic agent tripartite conjugates bound at the surface, inducing internalization of the tripartite conjugate (and thus the active agent). Alternatively, targeting moiety-ligand may be delivered to the target cell surface, followed by administration of anti-ligand-therapeutic agent.

In one aspect of the present invention, a targeting moiety-anti-ligand conjugate is administered in vivo; upon target localization of the targeting

moiety-anti-ligand conjugate (i.e., and clearance of this conjugate from the circulation), an active agent-ligand conjugate is parenterally administered. This method enhances retention of the targeting moiety-anti-ligand : ligand-active agent complex at the target cell (as compared with targeting moiety-ligand : anti-ligand : ligand-active agent complexes and targeting moiety-ligand : anti-ligand-active agent complexes). Although a variety of ligand/anti-ligand pairs may be suitable for use within the claimed invention, a preferred ligand/anti-ligand pair is biotin/avidin.

In a second aspect of the invention, radioiodinated biotin and related methods are disclosed. Previously, radioiodinated biotin derivatives were of high molecular weight and were difficult to characterize. The radioiodinated biotin described herein is a low molecular weight compound that has been easily and well characterized.

In a third aspect of the invention, a targeting moiety-ligand conjugate is administered in vivo; upon target localization of the targeting moiety-ligand conjugate (i.e., and clearance of this conjugate from the circulation), a drug-anti-ligand conjugate is parenterally administered. This two-step method not only provides pretargeting of the targeting moiety conjugate, but also induces internalization of the subsequent targeting moiety-ligand-anti-ligand-drug complex within the target cell. Alternatively, another embodiment provides a three-step protocol that produces a targeting moiety-ligand : anti-ligand : ligand-drug complex at the surface, wherein the ligand-drug conjugate is administered simultaneously or within a short period of time after administration of anti-ligand (i.e., before the targeting moiety-ligand-anti-ligand complex has been removed from the target cell surface).

In a fourth aspect of the invention, methods for radiolabeling biotin with technetium-99m, rhenium-186 and rhenium-188 are disclosed. Previously, biotin derivatives were radiolabeled with indium-111 for use in pretargeted immunoscintigraphy (for instance, Virzi et al., Nucl. Med. Biol. 18:719-26, 1991; Kalofonos et al., J. Nucl. Med. 31: 1791-96, 1990; Paganelli et al., Canc. Res. 51:5960-66, 1991). However, ^{99m}Tc is a particularly preferred radionuclide for immunoscintigraphy due to (i) low cost, (ii) convenient supply and (iii) favorable nuclear properties. Rhenium-186 displays chelating chemistry very similar to ^{99m}Tc, and is considered to be an excellent therapeutic radionuclide (i.e., a 3.7 day half-life and 1.07 MeV maximum particle that is similar to ^{131}I). Therefore, the claimed methods for technetium and rhenium radiolabeling of biotin provide numerous advantages.

The "targeting moiety" of the present invention binds to a defined target cell population, such as tumor cells. Preferred targeting moieties useful in this regard include antibody and antibody fragments, peptides, and hormones. Proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, anti-HER2, platelet binding proteins such as annexins, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting moieties. Also, anti-EGF receptor antibodies, which internalize following binding to the receptor and traffic to the nucleus to an extent, are preferred targeting moieties for use in the present invention to facilitate delivery of Auger emitters and nucleus binding drugs to target cell nuclei. Oligonucleotides, e.g., antisense oligonucleotides that are complementary to portions of target cell

nucleic acids (DNA or RNA), are also useful as targeting moieties in the practice of the present invention. Oligonucleotides binding to cell surfaces are also useful. Analogs of the above-listed targeting moieties that retain the capacity to bind to a defined target cell population may also be used within the claimed invention. In addition, synthetic targeting moieties may be designed.

Functional equivalents of the aforementioned molecules are also useful as targeting moieties of the present invention. One targeting moiety functional equivalent is a "mimetic" compound, an organic chemical construct designed to mimic the proper configuration and/or orientation for targeting moiety-target cell binding. Another targeting moiety functional equivalent is a short polypeptide designated as a "minimal" polypeptide, constructed using computer-assisted molecular modeling and mutants having altered binding affinity, which minimal polypeptides exhibit the binding affinity of the targeting moiety.

Preferred targeting moieties of the present invention are antibodies (polyclonal or monoclonal), peptides, oligonucleotides or the like. Polyclonal antibodies useful in the practice of the present invention are polyclonal (Vial and Callahan, Univ. Mich. Med. Bull., 20: 284-6, 1956), affinity-purified polyclonal or fragments thereof (Chao et al., Res. Comm. in Chem. Path. & Pharm., 9: 749-61, 1974).

Monoclonal antibodies useful in the practice of the present invention include whole antibody and fragments thereof. Such monoclonal antibodies and fragments are producible in accordance with conventional techniques, such as hybridoma synthesis, recombinant DNA techniques and protein synthesis. Useful monoclonal antibodies and fragments may be derived from any species (including humans) or may be

formed as chimeric proteins which employ sequences from more than one species. See, generally, Kohler and Milstein, Nature, 256: 495-97, 1975; Eur. J. Immunol., 6: 511-19, 1976.

Human monoclonal antibodies or "humanized" murine antibody are also useful as targeting moieties in accordance with the present invention. For example, murine monoclonal antibody may be "humanized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) or the complementarity determining regions thereof with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Some murine residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized targeting moieties are recognized to decrease the immunoreactivity of the antibody or polypeptide in the host recipient, permitting an increase in the half-life and a reduction in the possibility of adverse immune reactions.

An additional aspect of the present invention is directed to the use of targeting moieties that are monoclonal antibodies or fragments thereof that localize to an antigen that is recognized by the antibody NR-LU-10. Such monoclonal antibodies or fragments may be murine or of other non-human mammalian origin, chimeric, humanized or human.

NR-LU-10 is a 150 kilodalton molecular weight IgG2b monoclonal antibody that recognizes an approximately 40 kilodalton glycoprotein antigen expressed on most carcinomas. In vivo studies in mice using an antibody specific for the NR-LU-10 antigen revealed that such antibody was not rapidly

internalized, which would have prevented localization of the subsequently administered active-agent-containing conjugate to the target site.

NR-LU-10 is a well characterized pancarcinoma antibody that has been safely administered to over 565 patients in human clinical trials. The hybridoma secreting NR-LU-10 was developed by fusing mouse splenocytes immunized with intact cells of a human small cell lung carcinoma with P3 x 63/Ag8UI murine myeloma cells. After establishing a seed lot, the hybridoma was grown via in vitro cell culture methods, purified and verified for purity and sterility.

Radioimmunoassays, immunoprecipitation and Fluorescence-Activated Cell Sorter (FACS) analysis were used to obtain reactivity profiles of NR-LU-10. The NR-LU-10 target antigen was present on either fixed cultured cells or in detergent extracts of various types of cancer cells. For example, the NR-LU-10 antigen is found in small cell lung, non-small cell lung, colon, breast, renal, ovarian, pancreatic, and other carcinoma tissues. Tumor reactivity of the NR-LU-10 antibody is set forth in Table A, while NR-LU-10 reactivity with normal tissues is set forth in Table B. The values in Table B are obtained as described below. Positive NR-LU-10 tissue reactivity indicates NR-LU-10 antigen expression by such tissues. The NR-LU-10 antigen has been further described by Varki et al., "Antigens Associated with a Human Lung Adenocarcinoma Defined by Monoclonal Antibodies," Cancer Research, 44: 681-687, 1984, and Okabe et al., "Monoclonal Antibodies to Surface Antigens of Small Cell Carcinoma of the Lung," Cancer Research, 44: 5273-5278, 1984.

The tissue specimens were scored in accordance with three reactivity parameters: (1) the intensity of the reaction; (2) the uniformity of the reaction within the cell type; and (3) the percentage of cells

reactive with the antibody. These three values are combined into a single weighted comparative value between 0 and 500, with 500 being the most intense reactivity. This comparative value facilitates comparison of different tissues. Table B includes a summary reactivity value, the number of tissue samples examined and the number of samples that reacted positively with NR-LU-10.

Methods for preparing antibodies that bind to epitopes of the NR-LU-10 antigen are described in U.S. Patent No. 5,084,396. Briefly, such antibodies may be prepared by the following procedure:

- absorbing a first monoclonal antibody directed against a first epitope of a polyvalent antigen onto an inert, insoluble matrix capable of binding immunoglobulin, thereby forming an immunosorbent;
- combining the immunosorbent with an extract containing polyvalent NR-LU-10 antigen, forming an insolubilized immune complex wherein the first epitope is masked by the first monoclonal antibody;
- immunizing an animal with the insolubilized immune complex;
- fusing spleen cells from the immunized animal to myeloma cells to form a hybridoma capable of producing a second monoclonal antibody directed against a second epitope of the polyvalent antigen;
- culturing the hybridoma to produce the second monoclonal antibody; and
- collecting the second monoclonal antibody as a product of the hybridoma.

Table A

Organ/Cell Type Tumor	#Pos/ Exam	Intensity[a] Avg.	Range	Percent[b] Avg.	Range	Uniformity[c] Avg.	Range
Pancreas Carcinoma	6/6	3	3	100	100	2-3	2-3
Prostate Carcinoma	9/9	2.8	2-3	95	80-100	2	1-3
Lung Adenocarcinoma	8/8	3	3	100	100	2.2	1-3
Lung Small Cell Carcinoma	2/2	3	3	100	100	2	2
Lung Squamous Cell Carcinoma	8/8	2.3	2-3	73	5-100	1.8	1-3
Renal Carcinoma	8/9	2.2	2-3	83	75-100	1	1
Breast Adenocarcinoma	23/23	2.9	2-3	97	75-100	2.8	1-3
Colon Carcinoma	12/12	2.9	2-3	98	95-100	2.9	2-3
Malignant Melanoma Ocular	0/2	0	0	0	0	0	0
Malignant Melanoma	0/11	0	0	0	0	0	0
Ovarian Carcinoma	35/35	2.9	2-3	200	100	2.2	1-3
Undifferentiated Carcinoma	1/1	2	2	90	90	2	2
Osteosarcoma	1/1	2	2	20	20	1	1
Synovial Sarcoma	0/1	0	0	0	0	0	0
Lymphoma	0/2	0	0	0	0	0	0
Liposarcoma	0/2	0	0	0	0	0	0
Uterine Leiomyosarcoma	0/1	0	0	0	0	0	0

[a] Rated from 0-3, with 3 representing highest intensity.
[b] Percentage of cells stained within the examined tissue section.
[c] Rates from 0-3, with 3 representing highest uniformity.

Table B

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Adenoid		
Epithelium	3/3	433
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Mucus Gland	2/2	400
Adipose Tissue		
Fat Cells	0/3	0
Adrenal		
Zona Fasciculata Cortex	0/3	0
Zona Glomerulosa Cortex	0/3	0
Zona Reticularis Cortex	0/3	0
Medulla	0/3	0
Aorta		
Endothelium	0/3	0
Elastic Interna	0/3	0
Tunica Adventitia	0/3	0
Tunica Media	0/3	0
Brain-Cerebellum		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0
Purkenje's Cells	0/3	0
Brain-Cerebrum		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Brain-Midbrain		
Axons, Myelinated	0/3	0
Microglia	0/3	0
Neurons	0/3	0
Colon		
Mucosal Epithelium	3/3	500
Muscularis Externa	0/3	0
Muscularis Mucosa	0/3	0
Nerve Ganglia	0/3	0
Serosa	0/1	0
Duodenum		
Mucosal Epithelium	3/3	500
Muscularis Mucosa	0/3	0
Epididymis		
Epithelium	3/3	419
Smooth Muscle	0/3	0
Spermatozoa	0/1	0
Esophagus		
Epithelium	3/3	86
Mucosal Gland	2/2	450
Smooth Muscle	0/3	0
Gall Bladder		
Mucosal Epithelium	0/3	467
Smooth Muscle	0/3	0

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Heart		
Myocardium	0/3	0
Serosa	0/1	0
Ileum		
Lymph Node	0/2	0
Mucosal Epithelium	0/2	0
Muscularis Externa	0/1	0
Muscularis Mucosa	0/2	0
Nerve Ganglia	0/1	0
Serosa	0/1	0
Jejunum		
Lymph Node	0/1	0
Mucosal Epithelium	2/2	400
Muscularis Externa	0/2	0
Muscularis Mucosa	0/2	0
Nerve Ganglia	0/2	0
Serosa	0/1	0
Kidney		
Collecting Tubules	2/3	160
Distal Convoluted Tubules	3/3	500
Glomerular Epithelium	0/3	0
Mesangial	0/3	0
Proximal Convoluted Tubules	3/3	500

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Liver		
Bile Duct	3/3	500
Central Lobular Hepatocyte	1/3	4
Periportal Hepatocyte	1/3	40
Kupffer Cells	0/3	0
Lung		
Alveolar Macrophage	0/3	0
Bronchial Epithelium	0/2	0
Bronchial Smooth Muscle	0/2	0
Pneumocyte Type I	3/3	354
Pneumocyte Type II	3/3	387
Lymph Node		
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Mammary Gland		
Aveolar Epithelium	3/3	500
Duct Epithelium	3/3	500
Myoepithelium	0/3	0
Muscle Skeletal		
Muscle Fiber	0/3	0
Nerve		
Axon, Myelinated	0/2	0
Endoneurium	0/2	0
Neurolemma	0/2	0
Neuron	0/2	0
Perineurium	0/2	0

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Ovary		
Corpus Luteum	0/3	0
Epithelium	1/1	270
Granulosa	1/3	400
Serosa	0/3	0
Theca	0/3	0
Oviduct		
Epithelium	1/1	500
Smooth Muscle	0/3	0
Pancreas		
Acinar Cell	3/3	500
Duct Epithelium	3/3	500
Islet Cell	3/3	500
Peritoneum		
Mesothelium	0/1	0
Pituitary		
Adenohypophysis	2/2	500
Neurohypophysis	0/2	0
Placenta		
Trophoblasts	0/3	0
Prostate		
Concretions	0/3	0
Glandular Epithelium	3/3	400
Smooth Muscle	0/3	0

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Rectum		
Lymph Node	0/2	0
Mucosal Epithelium	0/2	0
Muscularis Externa	0/1	0
Muscularis Mucosa	0/3	0
Nerve Ganglia	0/3	0
Salivary Gland		
Acinar Epithelium	3/3	500
Duct Epithelium	3/3	500
Skin		
Apocrine Glands	3/3	280
Basal Layer	3/3	33
Epithelium	1/3	10
Follicle	1/1	190
Stratum Corneum	0/3	0
Spinal Cord		
Axons, Myelinated	0/2	0
Microglial	0/2	0
Neurons	0/2	0
Spleen		
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Trabecular Smooth Muscle	0/3	0

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Stomach		
Chief Cells	3/3	290
Mucosal Epithelium	3/3	367
Muscularis Mucosa/Externa	0/3	0
Parietal Cells	3/3	290
Smooth Muscle	0/3	0
Stromal Tissue		
Adipose	0/63	0
Arteriolar Smooth Muscle	0/120	0
Endothelium	0/120	0
Fibrous Connective Tissue	0/120	0
Macrophages	0/117	0
Mast Cells/Eosinophils	0/86	0
Testis		
Interstitial Cells	0/3	0
Sertoli Cells	3/3	93
Thymus		
Hassal's Epithelium	3/3	147
Hassal's Keratin	3/3	333
Lymphoid Cortex	0/3	0
Lymphoid Medulla	3/3	167
Thyroid		
C-cells	0/3	0
Colloid	0/3	0
Follicular Epithelium	3/3	500

Table B Cont'd

Organ/Cell Type	# Pos/Exam	Summary Reactivity
Tonsil		
Epithelium	1/3	500
Lymphoid Follicle-Central	0/3	0
Lymphoid Follicle-Peripheral	0/3	0
Mucus Gland	1/1	300
Striated Muscle	0/3	0
Umbilical cord		
Epithelium	0/3	0
Urinary Bladder		
Mucosal Epithelium	3/3	433
Serosa	0/1	0
Smooth Muscle	0/3	0
Uterus		
Endometrial Epithelium	3/3	500
Endometrial Glands	3/3	500
Smooth Muscle	0/3	0
Vagina/Cervix		
Epithelial Glands	1/1	500
Smooth Muscle	0/2	0
Squamous Epithelium	1/1	200

Consequently, monoclonal antibodies NR-LU-01, NR-LU-02 and NR-LU-03, prepared in accordance with the procedures described in the aforementioned patent, are exemplary targeting moieties useful in this aspect of the present invention.

Additional antibodies reactive with the NR-LU-10 antigen may also be prepared by standard hybridoma production and screening techniques. Any hybridoma clones so produced and identified may be further screened as described above to verify antigen and tissue reactivity.

Types of active agents (diagnostic or therapeutic) useful herein include toxins, anti-tumor agents, drugs and radionuclides. Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting protein. However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the toxin A chain-targeting protein conjugate, as compared to the corresponding holotoxin-targeting protein conjugate.

Preferred toxins in this regard include holotoxins, such as abrin, ricin, modeccin, Pseudomonas exotoxin A, Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of Pseudomonas exotoxin A, Diphtheria toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom

peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Extremely highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention. Also, toxin moieties that are charge-modified in accordance with the description of protein charge modification set forth below, e.g., succinic anhydride modification, also find utility in the practice of the present invention.

Preferred drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide and cis-platinum, as well as other conventional chemotherapeutics as described in Cancer: Principles and Practice of Oncology, 2d ed., V.T. DeVita, Jr., S. Hellman, S.A. Rosenberg, J.B. Lippincott Co., Philadelphia, PA, 1985, Chapter 14. A particularly preferred drug within the present invention is a trichothecene.

Trichothecenes are drugs produced by soil fungi of the class *Fungi imperfecti* or isolated from *Baccharus megapotamica* (Bamburg, J.R. Proc. Molec. Subcell. Biol. 8:41-110, 1983; Jarvis & Mazzola, Acc. Chem. Res. 15:338-395, 1982). They appear to be the most toxic molecules that contain only carbon, hydrogen and oxygen (Tamm, C. Fortschr. Chem. Org. Naturst. 31:61-117, 1974). They are all reported to act at the level of the ribosome as inhibitors of protein synthesis at the initiation, elongation, or termination phases.

There are two broad classes of trichothecenes: those that have only a central sesquiterpenoid structure and those that have an additional macrocyclic ring (simple and macrocyclic

trichothecenes, respectively). The simple trichothecenes may be subdivided into three groups (i.e., Group A, B, and C) as described in U.S. Patent Nos. 4,744,981 and 4,906,452 (incorporated herein by reference). Representative examples of Group A simple trichothecenes include: Scirpene, Roridin C, dihydrotrichothecene, Scirpen-4, 8-diol, Verrucarol, Scirpentriol, T-2 tetraol, pentahydroxyscirpene, 4-deacetylneosolaniol, trichodermin, deacetylcalonectrin, calonectrin, diacetylverrucarol, 4-monoacetoxyscirpenol, 4,15-diacetoxyscirpenol, 7-hydroxydiacetoxyscirpenol, 8-hydroxydiacetoxy-scirpenol (Neosolaniol), 7,8-dihydroxydiacetoxyscirpenol, 7-hydroxy-8-acetyldiacetoxyscirpenol, 8-acetylneosolaniol, NT-1, NT-2, HT-2, T-2, and acetyl T-2 toxin. Representative examples of Group B simple trichothecenes include: Trichothecolone, Trichothecin, deoxynivalenol, 3-acetyldeoxynivalenol, 5-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, Nivalenol, 4-acetylnivalenol (Fusarenon-X), 4,15-idacetylnivalenol, 4,7,15-triacetylnivalenol, and tetra-acetylnivalenol. Representative examples of Group C simple trichothecenes include: Crotocol and Crotocin. Representative macrocyclic trichothecenes include Verrucarin A, Verrucarin B, Verrucarin J (Satratoxin C), Roridin A, Roridin D, Roridin E (Satratoxin D), Roridin H, Satratoxin F, Satratoxin G, Satratoxin H, Vertisporin, Mytoxin A, Mytoxin C, Mytoxin B, Myrotoxin A, Myrotoxin B, Myrotoxin C, Myrotoxin D, Roritoxin A, Roritoxin B, and Roritoxin D. In addition, the general "trichothecene" sesquiterpenoid ring structure is also present in compounds termed "baccharins" isolated from the higher plant *Baccharis megapotamica*, and these are described in the literature, for instance as disclosed by

Jarvis et al. (Chemistry of Alleopathy, ACS Symposium Series No. 268: ed. A.C. Thompson, 1984, pp. 149-159).

Experimental drugs, such as mercaptopurine, N-methylformamide, 2-amino-1,3,4-thiadiazole, melphalan, hexamethylmelamine, gallium nitrate, 3% thymidine, dichloromethotrexate, mitoguazone, suramin, bromodeoxyuridine, iododeoxyuridine, semustine, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, N,N'-hexamethylene-bis-acetamide, azacitidine, dibromodulcitol, Erwinia asparaginase, ifosfamide, 2-mercaptoethane sulfonate, teniposide, taxol, 3-deazauridine, soluble Baker's antifol, homoharringtonine, cyclocytidine, acivicin, ICRF-187, spiromustine, levamisole, chlorozotocin, aziridinyl benzoquinone, spirogermanium, aclarubicin, pentostatin, PALA, carboplatin, amsacrine, caracemide, iproplatin, misonidazole, dihydro-5-azacytidine, 4'-deoxy-doxorubicin, menogaril, triciribine phosphate, fazarabine, tiazofurin, teroxirone, ethiofos, N-(2-hydroxyethyl)-2-nitro-1H-imidazole-1-acetamide, mitoxantrone, acodazole, amonafide, fludarabine phosphate, pibenzimol, didemnin B, merbarone, dihydrolenperone, flavone-8-acetic acid, oxantrazole, ipomeanol, trimetrexate, deoxyspergualin, echinomycin, and dideoxycytidine (see NCI Investigational Drugs, Pharmaceutical Data 1987, NIH Publication No. 88-2141, Revised November 1987) are also preferred.

Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Radionuclides are well-known in the art and include ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}Y, ^{88}Y, ^{97}Ru, ^{100}Pd, ^{101m}Rh, ^{119}Sb, ^{128}Ba, ^{197}Hg, ^{211}At, ^{212}Bi, ^{153}Sm, ^{169}Eu, ^{212}Pb, ^{109}Pd, ^{111}In, ^{67}Ga, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{166}Ho and ^{18}F. Preferred therapeutic radionuclides include ^{188}Re, ^{186}Re,

^{203}Pb, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au, ^{166}Ho and ^{199}Ag or ^{177}Lu.

Other anti-tumor agents, e.g., agents active against proliferating cells, are administrable in accordance with the present invention. Exemplary anti-tumor agents include cytokines, such as IL-2, tumor necrosis factor or the like, lectin inflammatory response promoters (selectins), such as L-selectin, E-selectin, P-selectin or the like, and like molecules.

Ligands suitable for use within the present invention include biotin, haptens, lectins, epitopes, dsDNA fragments, enzyme inhibitors and analogs and derivatives thereof. Useful complementary anti-ligands include avidin (for biotin), carbohydrates (for lectins) and antibody, fragments or analogs thereof, including mimetics (for haptens and epitopes) and zinc finger proteins (for dsDNA fragments) and enzymes (for enzyme inhibitors). Preferred ligands and anti-ligands bind to each other with an affinity of at least about $k_D \geq 10^9$ M.

One component to be administered in a preferred two-step pretargeting protocol is a targeting moiety-anti-ligand or a targeting moiety-ligand conjugate. In three-step pretargeting, a preferred component for administration is a targeting moiety-ligand conjugate. Accordingly, the present invention provides an article of manufacture which includes packaging material and a targeting moiety-anti-ligand (e.g., streptavidin) conjugate or a targeting moiety-ligand (e.g., biotin) conjugate contained within the packaging material, wherein the conjugate is capable of localizing at a target site upon administration to a mammalian recipient, and the ligand or anti-ligand retains the capability to bind to the complementary ligand/anti-ligand binding pair member, and wherein the packaging material includes a label that identifies the targeting moiety component of the conjugate,

identifies the ligand or anti-ligand component of the conjugate and indicates an appropriate use of the conjugate in human recipients.

The packaging material indicates whether the conjugate is limited to investigational use or identifies an indication for which the conjugate has been approved by the U.S. Food and Drug Administration or other similar regulatory body for use in humans. The packaging material may also include additional information including the amount of conjugate, the medium or environment in which the conjugate is dispersed, if any, lot number or other identifier, storage instructions, usage instructions, a warning with respect to any restriction upon use of the conjugate, the name and address of the company preparing and/or packaging the conjugate, and other information concerning the conjugate.

The targeting moiety-ligand conjugate or targeting moiety-anti-ligand conjugate is preferably contained within a vial which allows the first conjugate to be transported prior to use. Such conjugate is preferably vialed in a sterile, pyrogen-free environment. Alternatively, the conjugate may be lyophilized prior to packaging. In this circumstance, instructions for preparing the lyophilized conjugate for administration to a recipient may be included on the label.

If the targeting moiety of the targeting moiety-ligand or targeting moiety-anti-ligand conjugate is a monoclonal antibody or antibody fragment of murine origin, a host immune response will be generated against the antibody component or the component conjugated to the antibody component upon conjugate administration to a mammal of differing species. B-cells recognize antigen by a mechanism whereby early B-cells bind the circulating foreign protein. A signal is transduced via recognition of IgM, and the

B-cell is stimulated to multiply and form memory cells. Upon subsequent binding of antigen, higher affinity B-cells are selected and produced in higher quantity. B-cell antigen recognition is therefore independent of MHC cell presentation.

Nonspecific uptake into normal tissues results in catabolism of the conjugate to peptides by macrophages or other antigen presenting cells with presentation of the peptides to immunocompetent T-cells. These peptides could then be recognized as non-self with respect to the recipient by T-cell dependent or independent mechanisms and augment the antibody response generated by B-cells. Biodistribution, serum clearance, normal organ accumulation and excretion impact the degree of conjugate immunogenicity and, therefore, the ability to administer conjugate multiple times to a recipient.

To reduce the immunogenicity of targeting moiety-ligand or targeting moiety-anti-ligand conjugates that employ antibody or antibody fragment targeting moieties (e.g., Fab or Fab' fragments), chimeric, humanized or human antibodies or fragments are used instead of their murine or other mammalian counterparts. Antibodies or fragments thereof exhibiting more human character are less likely to be recognized by the human immune system as non-self entities. Such antibodies or fragments are therefore generally less immunogenic than antibodies or fragments exhibiting less human character.

Chimeric antibodies (i.e., antibodies characterized by human constant regions and, generally, murine variable regions) are prepared in accordance with known procedures described in U.S. Patent No. 4,816,397, issued to Boss et al. and U.S. Patent No. 4,816,567, issued to Cabilly et al. Antibody humanization techniques (i.e., methods for the preparation of antibodies characterized by human

constant regions and variable framework regions and, generally, murine complementarity determining regions with some retained murine framework residues) are discussed in United Kingdom Patent No. 2,188,638 issued to Winter; PCT Patent Application No. WO92/11018 applied for by Protein Design Labs; PCT Patent Application No. WO92/04381 applied for by Scotgen Limited; and WO92/22653 applied for by Genentech. The use of transgenic mouse technology to form human antibodies (i.e., wholly human antibodies) has been reported. See, for example, Zebedee et al., PNAS, 89: 3175, 1992. Human or humanized antibodies, exhibiting more human character than chimeric antibodies, are more preferred.

Monoclonal antibody fragments (e.g., Fv, Fab, $F(ab')_2$) or other antigen or receptor targeting vehicles (e.g., molecular recognition units (generally less than 5 kD in molecular weight), lymphokines or factors such as epidermal growth factor), rather than whole monoclonal antibodies, may be employed as the targeting moieties of the present invention. Antibody fragments are lower in molecular weight (generally ranging from about 25-30 kD (e.g., single chain Fv as described, for example, in Biochem., 31(6): 1579, 1991) to about 45-50 kD (e.g., Fab) to about 100-105 kD ($F(ab')_2$)) than their whole antibody counterparts (generally ranging from about 150-160 kD (e.g., IgG) to about 900 kD (e.g., IgM)). Also, antibody fragments generally exhibit shorter serum half-lives in comparison to their whole antibody counterparts. A shorter serum half-life is believed to be related to reduced immunogenicity. In addition, the antibody fragment, presents a smaller and more rapidly excreted immunogenic target and, therefore, generally exhibits less immunogenicity than the larger and slower excreting immunogen, the whole antibody. Also, the Fc portion of an antibody generally exhibit more

immunogenicity than the Fab portion thereof. Antibody fragments offer the additional advantage faster, albeit less efficient, accretion to target sites, thereby enabling the time between pretargeting protocol agent administrations to be reduced. For example, a pretargeting protocol which employs Fab-streptavidin conjugate; clearing agent and active agent-biotin conjugate may be accomplished in mice by administering clearing agent at from about 2 to about 6 hours post-Fab-streptavidin conjugate administration, followed by active agent-biotin administration at about 1 hour thereafter.

On a percent initial dose/gram (%ID/g) basis, whole antibody generally exhibits a higher %ID/g than antibody fragment. The molecular weight of Fab fragments for example is about one-third that of whole antibody, however. Consequently, the moles of the lower molecular weight fragment or fragment-ligand or fragment-anti-ligand molecules accreting to a target site is generally greater than the number of moles of whole antibody or whole antibody-ligand or whole antibody-anti-ligand molecules so accreting, thereby affording a higher concentration of ligand or anti-ligand molecules associated with the target site. Antibody fragmentation techniques are known in the art and routinely practiced.

For similar reasons to those set forth above for antibody fragments, other small molecule targeting moieties generally exhibit less immunogenicity than larger molecular weight immunogens, such as whole antibody. For example, somatostatin and derivatives thereof such as octreotide and the like, annexins, cytokines such as IL-6, and the like are useful as targeting moieties in the practice of the present invention.

As discussed above, immunogenicity is impacted by biodistribution, serum clearance, normal organ

accumulation and excretion, which parameters are somewhat interrelated. Generally, decreases in serum clearance and excretion will result in increased immunogenicity, because the recipient remains exposed to the immunogen for a longer period of time. Normal organ accumulation (non-specific uptake) results in antigen presentation of macrophage-metabolized conjugate to T-cells. Conjugation of the targeting moiety to molecules that enhance RES uptake of the conjugate also generally increases immunogenicity.

Whole conjugate or individual conjugate components of targeting moiety-anti-ligand or targeting moiety-ligand conjugates may be chemically modified to reduce the immunogenicity thereof. Chemical modification may be employed so long as the targeting moiety retains the capability to localize to target sites and the ligand or anti-ligand retains the ability to bind to the complementary member of the ligand/anti-ligand pair with high affinity.

One example of a suitable chemical modification is PEGylation. Enzymes covalently attached to methoxy-polyethylene glycol (5000 molecular weight), PEGylated enzymes, have been shown generally to be non-immunogenic. See, for example, Abuchowski et al., "Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase," J. Biol. Chem., 252: 3581 (1977). Such PEGylated enzymes retained enzyme activity and exhibited an extended serum half-life.

Other proteinaceous moieties (e.g., proteinaceous ligands or anti-ligands, such as the anti-ligand streptavidin, antibodies, antibody fragments and other proteinaceous targeting moieties) may be PEGylated by substantially the same procedures by which enzymes are PEGylated. One useful PEGylation procedure employs previously derivatized cyanuric chloride-PEG (available from Sigma Chemical Co., St. Louis,

Missouri) which reacts with protein amine groups and proceeds, for example, as follows:

- Protein or protein-bearing conjugate is buffer exchanged into 0.1M borate, pH 9.2;
- Cyanuric chloride-PEG is added at an appropriate stoichiometric level (excess of PEG in comparison to conjugate) and the mixture is incubated for one hour at room temperature; and
- the PEGylated product is purified by anion exchange chromatography, wherein the product passed through while underivatized protein or protein-bearing conjugate bound to the matrix.

Also, non-proteinaceous moieties having appropriate functional groups may also be PEGylated.

In general, the PEGylation procedure is as follows: a hydroxyl group of poly(ethyleneglycol) monomethylether is activated to form an active ester for reaction with an amine or other appropriate functional group on a proteinaceous or non-proteinaceous moiety. For example, poly(ethyleneglycol) monomethylether, commercially available from Sigma Chemical Company, St. Louis, Missouri, is reacted to succinic anhydride to form poly(ethyleneglycol) monomethylether mono succinic acid. The acid is reacted with DCC and N-hydroxy succinimide to form the product ester.

Alternatively, other activating groups may be employed to produce a poly(ethyleneglycol) molecule amenable to conjugation with proteins, peptides or other moieties having suitable functional groups. Exemplary of such activating groups are cyanuric chloride (described in Anal. Biochem., 165: 114 (1987) and J. Biol. Chem., 252: 3578 (1977)); carbonate active esters such as para-nitrophenylcarbonate, succinimidyl carbonate or the like (see, generally, Appl. Biochem. Biotech., 11: 141 (1985)); other

activated carbonates such as imidazolyl carbonate (Anal. Biochem., 131: 25 (1983)).

The offering ratio of PEG to protein impacts the results, with increased PEG rendering the protein both non-antigenic (i.e., unable to react with antibodies formed against the native protein) as well as non-immunogenic. For streptavidin, for example, offering ratios of PEG:streptavidin and PEG:antibody will range between from about 5:1 to about 10:1.

The ability of PEGylated ligands and PEGylated anti-ligands to bind to the complementary member of the ligand/anti-ligand pair is tested in accordance with know procedures for testing ligand/anti-ligand binding affinity. See Example XI(E)(4), for example. Also, immune response profiles against the ligand or anti-ligand may be developed. Likewise, the ability of PEGylated proteinaceous targeting moiety to bind to target may be tested in accordance with known procedures for testing targeting moiety/target cell or target antigen binding. See Example XI(E)(5), for example.

Charge modification of proteinaceous targeting moieties and conjugates containing such targeting moieties and diagnostically or therapeutically active agents is discussed in published European Patent Application No. EP 329,184. One possible result of charge modification is an extension of serum half-life of the conjugate, which increases the bioavailability of the active agent-containing conjugate for the target site. The adverse impact of increased serum retention time, exposure of non-target tissue to the active agent, is addressed in the European Patent application through a concomitant reduction in non-specific targeting moiety localization. In the practice of the present invention, the decoupling of the pharmacokinetics of the targeting moiety and the active agent solves the problem.

Most antibodies and fragments thereof exhibit neutral or basic pI. Also, antibodies when fragmented exhibit characteristic changes in isoelectric point. Typically, fragments are basic compared to whole antibody, with the general exception that antibodies of the gamma-2b subclass undergo an acidic shift upon fragmentation. Fragments exhibiting a basic shift are characterized by greater kidney localization and shorter serum half life. Such fragments, being faster clearing and less immunogenic than their whole antibody counterparts, are particularly useful in diagnostic pretargeting protocols with or without clearing agent administration. Such fragments can also be used in therapeutic pretargeting protocols of the present invention.

Preferred charge modification in accordance with the present invention involves treatment of a proteinaceous targeting moiety and/or a proteinaceous ligand and/or a proteinaceous anti-ligand with a anion-forming reagent to provide a charge-modified conjugate exhibiting an acidic shift in isoelectric point. Preferably, the shift in isoelectric point is one-tenth of a pH unit or greater. Generally, charge-modified proteins exhibit a serum half-life that is at least 10% greater than the half-life of native proteins. A 50% or greater increase in half-life is not uncommon following charge modification to a protein.

Charge modification introducing more negative charge on a proteinaceous targeting moiety or on both components of a proteinaceous ligand- or anti-ligand-targeting moiety conjugate is a reduction in non-specific binding. Such a reduction directly reduces the ability of macrophages to metabolize and present peptides to immunocompetent cells, thereby resulting in reduced immunogenicity as well as slower metabolism and elimination of charge-modified conjugate. In

addition, peptides with a more homogenous charge distribution are characterized by an exposed surface of increased uniformity and are therefore less recognized by T-cell dependent mechanisms in a manner similar to polymers with repetitive sequences which are also less immunogenic.

Anion-forming agents useful in the practice of the present invention are structured to react with functional groups of the protein to be charge-modified and incorporate a negatively charged group to impart an acidic shift in the pI of the protein to be charge-modified. Preferred anion-forming agents useful in the practice of the present invention are structured to react with primary amines on lysine residues of the protein to be charge modified. Such anion-forming agents include active esters (carboxylic acid and imidate), maleimides and anhydrides. Preferred active esters include N-hydroxysuccinimidyl, thiophenyl, 2,3,5,6-tetrafluorophenyl, and 2,3,5,6,-tetrafluorothiophenyl esters. Derivatization of other protein residues may also be employed in the practice of the present invention (e.g., derivatization of arginine residues with glyoxal, phenyl glyoxal or cyclohexanedione). Negatively charged groups which may be used to impart an acidic shift to proteinaceous targeting moieties, ligands or anti-ligands include phosphates, phosphonates, sulfates, nitrates, borates, silicates, carbonates, and carboxyl groups such as native carboxyl groups or carboxyl groups generated from an anhydride during the reaction of the anion-forming agent with the protein.

Useful anion-forming agents include compounds incorporating an anhydride and/or at least one COOH group, such as succinic anhydride, other cyclic acid anhydrides, phthalic anhydride, maleic anhydride, N-ethyl maleimide substituted with carboxyl groups, aliphatic anhydrides (e.g., acetic anhydride),

aromatic anhydrides, pH-reversible anhydrides (e.g., citraconic anhydride and dimethyl maleic anhydride), alpha halo acids such as bromoacetate and iodoacetate, and diacids or triacids substituted with a functional group that reacts with an amino acid on a protein to be charge-modified.

For example, succinic anhydride is dissolved in DMSO or another dry organic solvent at a concentration of 40 mg per 200 microliters. This succinic anhydride solution (or a dilution thereof up to 2.5 ml in anhydrous DMSO, 1.73×10^{-2}M) is added to a protein (e.g., antibody, antibody fragment, ligand, anti-ligand or conjugate containing one or more of these components) solution (e.g., 3-5 mg/ml in carbonate/bicarbonate buffer, pH 8.5-9.0) at molar ratios of protein to succinic anhydride of 1:5, 1:10 and 1:25 (with higher molar ratios preferred). The reaction is carried out at room temperature for 15-30 minutes. After reaction completion, succinic acid is removed by ultrafiltration or by gel filtration. The degree of isoelectric shift is determined by isoelectric focusing.

The ability of charge-modified ligands and charge-modified anti-ligands to bind to the complementary member of the ligand/anti-ligand pair is tested in accordance with known procedures for testing ligand/anti-ligand binding affinity. Likewise, the ability of charge-modified proteinaceous targeting moiety to bind to target may be tested in accordance with known procedures for testing targeting moiety/target cell or target antigen binding.

Another chemical modification that may be employed in the practice of the present invention is polymer derivatization. Polymers exhibit a repetitive structure and have, in some instances, been found to be poorly immunogenic or non-immunogenic. For example, highly polymerized molecules of bacterial

origin are T-cell independent, and a low affinity response can be generated against such molecules. Such polymers may be conjugated to either or both of the components of the targeting moiety-ligand or targeting moiety-anti-ligand conjugates of the present invention, rendering them less immunogenic.

Generally, polymers having a small repetitive unit, a long serum half-life and relatively low molecular weight are useful in the practice of the present invention. Exemplary polymers useful in the practice of the present invention are alpha-amino acid polymers, vinyl polymers and dextrans.

The immunogenicity of poly-alpha-amino acid polymers depends upon the structure of the polymer and upon the immunized species. In general, the more structurally homogeneous a molecule is, the less immunogenic it is. While antigens having one determinant may elicit a delayed type hypersensitivity, more than one determinant is generally required to elicit an antibody response. Homopolymers of alpha-amino acids are therefore generally non-immunogenic. Exemplary polymers of this type include poly-D-glutamic acid, poly-D-alanine, poly-glycine, poly-D-aspartic acid, poly-D-lysine, poly-D-proline, and poly-D-hydroxyproline. Copolymers of two amino acids are more immunogenic than homopolymers and polymers formed of greater numbers of amino acids exhibit even greater immunogenicity. Consequently, homopolymers are preferred for the practice of the present invention.

Generally, higher molecular weight molecules are more likely to be immunogenic than their lower molecular weight counterparts; however, lower molecular weight molecules may be rendered immunogenic by conjugation to an immunogen. Consequently, the alpha-amino acid homopolymers useful in the practice of the present invention range in molecular weight

from about 5 kD to about 200 kD, with from about 10 kD to about 50 kD preferred.

In a manner similar to that described above for charge-modified conjugates, alpha-amino acid homopolymers exhibiting a high net electric charge are generally characterized by low immunogenicity. The high charge interferes with the presentation of the homopolymer-derivatized conjugates to immunocompetent cells.

Generally, D-amino acids are less immunogenic than L-amino acids. D-amino acids may exhibit some immunogenicity, but such immunogenicity has been shown to occur only at very low doses because D-amino acids are highly efficient in inducing immune system tolerance. Also, D-amino acids are poorly metabolized and, therefore, are characterized by a long serum half-life. Homopolymers incorporating D-amino acids are preferred for use in the practice of the present invention.

Quaternary structure of alpha-amino acid polymers is of most importance with regard to immunogenicity, while antigenicity is highly influenced by secondary structure. Consequently, the most immunogenic portion of the polymer are antigenic determinants expressed on the surface of the quaternary structure and therefore exposed to the environment of the polymer. If such exposed polymer portions correspond to exposed portions of the polymer-containing conjugate and those exposed portions are non-immunogenic, the conjugate is likely to be less immunogenic than an non-polymer-containing conjugate.

Targeting moieties, such as antibodies or antibody fragments, ligands or anti-ligands may be derivatized with homopolymers of alpha-amino acids by known procedures. The ability of alpha amino acid homopolymer-derivatized ligands and polymer-derivatized anti-ligands to bind to the complementary

member of the ligand/anti-ligand pair is tested in accordance with known procedures for testing ligand/anti-ligand binding affinity. This procedure is based on ELISA techniques in which the derivatized molecule is tested for binding in a competitive assay against a non-derivatized molecule. Likewise, the ability of homopolymer-derivatized targeting moiety to bind to target may be tested in accordance with similar procedures for testing targeting moiety/target cell or target antigen binding.

Vinyl polymers are nondegradable under physiological conditions and, as a result, behave similarly to poly-D-amino acids and some natural polysaccharides. Exemplary vinyl polymers useful in the practice of the present invention are polyvinylpyrrolidone (similar to peptide polymers with regard to substituted amino groups and heterocyclic rings), poly-methacrylic acid-2-dimethylaminoethyl methacrylate (a polyampholyte with protein-like molecular charge distribution and glutamic acid-lysine copolymer-like structure) as well as polymethacrylic acid and polyvinylamine (fully charged vinyl analogs of homopolymers of glutamic acid and lysine, respectively) and the homopolymer of N-(2-hydroxypropyl)methacrylamide (HPMA). Only small doses of these vinyl polymers engendered an immune response. Higher doses were tolerogenic.

Targeting moieties, such as antibodies or antibody fragments, ligands or anti-ligands may be derivatized with vinyl polymers by known procedures. Vinyl polymers generally exhibit alcohol and carboxylic acid functionalities. In general, the vinyl polymer derivatization procedure is as follows: vinyl polymer carboxylic acid functional group is converted to active ester form through the use of reactive alcohols or phenols in the presence of water soluble carbodiimides; and the active ester is reacted with

appropriate functional groups of the targeting moiety, ligand or the anti-ligand to afford the product conjugates. This procedure can be conducted in either a one pot, single step manner or in a stepwise fashion as described above using coupling techniques that are known in the art.

The ability of vinyl polymer-derivatized ligands and vinyl polymer-derivatized anti-ligands to bind to the complementary member of the ligand/anti-ligand pair is tested in accordance with known procedures for testing ligand/anti-ligand binding affinity. Likewise, the ability of vinyl polymer-derivatized targeting moiety to bind to target may be tested in accordance with known procedures for testing targeting moiety/target cell or target antigen binding.

Dextrans are naturally occurring homopolymers which are formed as exopolysaccharides by some Gram-negative bacteria. Dextrans include alpha-D-glucose residues linked by glycosidic linkages selected from α(1->6), α(1->2), α(1->3) and α(1->4), with the majority of such linkages being α(1->6). The degree of branching of the alpha-D-glucose chains in the dextran structure is the basis of classification of dextrans as class I (characterized by a linear backbone of α(1->6) linkages with small side chains attached via one of the other linkage types or class II (characterized by more complicated structure, with an alternating α(1->3) and α(1->6) backbone).

Dextrans are hydrophilic, water soluble molecules and are not easily degraded in biological systems. Dextrans with a molecular weight above 90,000 are generally immunogenic in man. Lower molecular weight dextrans are not immunogenically active (i.e., generally are unable to induce either an immune response or tolerance). Low molecular weight dextrans (< 10,000 daltons) exhibit immunogenicity when conjugated to proteinaceous immunogens, however.

Dextrans useful in the practice of the present invention are characterized by molecular weights ranging from about 5 kD to about 200 kD, with from about 10 kD to about 50 kD preferred. Dextrans of varying molecular weights are commercially available from Sigma Chemical Co., St. Louis, Missouri, for example.

Targeting moieties, such as antibodies or antibody fragments, ligands or anti-ligands may be derivatized with dextrans by known procedures. In general, the dextran derivatization procedure is as follows: lysine-derivatized dextran polymers (commercially available from Sigma Chemical Co.) are activated by reacting a lysine residue thereof with the bifunctional reagent SMCC (commercially available from Pierce Chemical Co.), preferably at room temperature for 0.5 to 1.0 hours in a 0.1M sodium borate buffer, pH 8.0-8.5; the activated dextran polymer contains a maleimide group available for conjugation with endogenous sulfhydryl groups of targeting moieties generated by reaction of the targeting moiety with DTT, for example. Alternative functional groups may be introduced for conjugation with non-sulfhydryl-bearing targeting moieties, ligands or anti-ligands in accordance with procedures that are known in the art.

The ability of dextran-derivatized ligands and dextran-derivatized anti-ligands to bind to the complementary member of the ligand/anti-ligand pair is tested in accordance with know procedures for testing ligand/anti-ligand binding affinity. Likewise, the ability of dextran-derivatized targeting moiety to bind to target may be tested in accordance with known procedures for testing targeting moiety/target cell or target antigen binding.

Different chemical modification procedures may be employed in tandem. That is, one conjugate component could be rendered less immunogenic using one chemical

modification procedure, while another conjugate component is rendered less immunogenic using another. For example, the targeting moiety could be rendered less immunogenic via charge modification with succinic anhydride, while a ligand or anti-ligand is rendered less immunogenic via class I dextran derivatization. Alternatively, both conjugate components could be rendered less immunogenic using the same chemical modification procedure, which modification preferably is conducted following conjugation.

For antibody targeting moiety conjugates, antibodies with increased human character may be employed along with chemical modification of the ligand or anti-ligand. For monoclonal antibody-streptavidin conjugates, for example, the streptavidin portion may be PEGylated while the monoclonal antibody portion may be humanized. In this case, the chemical modification is preferably conducted prior to conjugation.

The chemically-modified conjugates useful in the present invention generally exhibit longer serum half-lives than their non-chemically-modified counterparts. In prior art processes wherein active agent is conjugated to the targeting moiety, such extended half-lives were desirable to increase the bioavailability of the active agent-containing conjugate to the target site. The drawback of an extended serum half-life is increased non-target site exposure to the active agent. The present invention addresses this drawback in that the kinetics of the active agent is decoupled from that of the targeting moiety. Also, the optional clearing agent of the present invention provides the basis for removing targeting moiety-ligand conjugate or targeting moiety-anti-ligand conjugate from the recipient regardless of the extended half-life of the conjugate.

Alternatively or concurrently, immunogenicity-reducing modification of the tertiary structure of a proteinaceous immunogen may be employed in the practice of the present invention. Such tertiary structure modification is preferably conducted by recombinant techniques to reduce the number or immunogenicity of immunogenic sites on the proteinaceous immunogen. A preferred modification involves altering the charge of surface amino acids from a net negative charge to a neutral charge. One method to accomplish this preferred modification is surface amino acid substitution conducted in a manner to substantially preserve the tertiary structure of the protein.

Another method to decrease the immunogenicity of administered conjugates is to administer an immunosuppressive agent before, during or after conjugate administration. Preferably, the immunosuppressive agents of the present invention are administered before, during and after conjugate administration. Immunosuppressive agents, such as cyclosporin A, which is commercially available from Sandoz Pharmaceuticals Corporation in various formulations, are typically non-targeted, systemically acting agents that generally reduce the recipient's immune response to non-self antigens.

Cyclosporin A is a neutral, hydrophobic cyclic peptide of 11 amino acids exhibiting a highly selective ability to inhibit activation of T-cells. This drug molecule is highly lipid soluble and, as a result, distributes in both the vascular and the extravascular physiological compartments, but does not penetrate the blood-brain barrier. Cyclosporin A is extensively metabolized (17+ metabolites, none active) and is excreted via the bile and feces. Unlike cytotoxic immunosuppressants, therapeutic concentrations of cyclosporin A do not cause

myelosuppression. Cyclosporin A can also restore the sensitivity of cell lines and experimental tumors that are resistant to several cancer therapeutics.

Cyclosporin A has been tested in humans and approved by the U.S. Food and Drug Administration for the prophylaxis of organ rejection in kidney, liver and heart allogenic transplants and in the treatment of chronic rejection in patients previously treated with other immunosuppressive agents. Cyclosporin A has also been tested in humans for the ability to decrease the immunogenicity of murine antibodies. The results of some experiments involving the impact of cyclosporin A on human-anti-mouse antibody (HAMA) are set forth in Ledermann et al., "Repeated Antitumor Antibody Therapy in Man with Suppression of the Host Response by Cyclosporin A" and in Example XX. As the article and the material presented in Example XX indicate, cyclosporin A has utility in managing HAMA.

Based upon the anti-organ transplant rejection and anti-HAMA data, cyclosporin A is a candidate for immunosuppression of human anti-streptavidin antibody (HASA) as well as for immunosuppression of conjugates incorporating an immunogenic targeting moiety. Cyclosporin A is commercially available in 25 mg soft gelatin capsules, as an oral solution of 100mg/mL, and in 5 ml sterile ampules containing 50 mg/ml for intravenous administration.

A dosing regimen for cyclosporin A to suppress the recipient's immune response to non-self moieties involves one or more pre-immunogen administration doses ranging from about 5 to about 15 mg/kg/day taken orally and ranging from about 1.5 to about 5.5 mg/kg/day for intravenous and daily post-immunogen administrations thereof for approximately 7 days weeks. Of course, the exact dosing regimen and the doses employed therein will be selected by the attending physician on a patient-by-patient basis

depending on a variety of factors known to practicing attending physicians.

Other immunosuppressive agents have shown utility in suppressing a recipient's response to non-self antigens. Preferably, immunosuppressive agents useful in the practice of the present invention exhibit one or more of the following characteristics: low toxicity; high efficacy; and the like. For example, azathioprine is known to directly inhibit B cell function and is approved for human use. Cyclophosphamide is also approved for human use.

Exemplary immunosuppressive agents include verapamil, mycophenolic acid, transforming growth factor-beta, deoxyspergualin, FK506, rapamycin, immunophilins such as FK binding protein 12, peptide derivatives of non-erythroid spectin, fluorinated cyclosporin analogs, such as those discussed in U.S. Patent No. 5,227,467, and combinations thereof. Clearing agents, as described below, are also useful as immunosuppressive agents in the practice of the present invention.

Deoxyspergualin, FK506, and verapamil have been tested in human clinical trials and exhibited the ability to suppress rejection following organ transplantation. Verapamil and deoxyspergualin have also been shown to modulate murine immune response to foreign proteins. In addition, deoxyspergualin has been shown to exhibit anti-neoplastic activity and is currently under evaluation in human clinical trials for that indication. Preferably, immunosuppressive agent is administered both prior to and following administration of the immunogen. Again, the exact immunosuppressive agent dosing regimen and the doses employed therein for suppressing the immune response of a recipient to administered targeting moiety-ligand or targeting moiety-anti-ligand conjugate will be selected by the attending physician on a patient-by-

patient basis depending on a variety of factors known to practicing attending physicians.

Immunosuppressive agents, characterized by one or more of the following: short serum half-life; low therapeutic index; high cost; and significant dose-related toxicity, are preferably employed in liposomal or particulate form for use in the practice of the present invention. Sustained release dosage forms of this type will extend the serum half-lives of the immunosuppressive agents encapsulated therein. The extended serum half-life will increase the bioavailability of the immunosuppressive agent to reduce the recipient's response to administered targeting moiety-ligand or targeting moiety-anti-ligand conjugate. Such dosage forms should also permit fewer administrations of immunosuppressive agent and/or a lower total immunosuppressive agent dose to constitute effective immunosuppressive regimens. Fewer administrations and/or lower total agent doses will reduce the cost of the protocol.

Also, sustained release dosage forms facilitate release of immunosuppressive agents over time, thereby decreasing the maximum exposure of the recipient to that agent. That is, the immunosuppressive agent recipient is not exposed to a large initial dose, rather the recipient is exposed to a gradually increasing dose to one or more steady state values over the course of the regimen, which steady state values are generally lower than the initial dose given in a protocol incorporating administration of free drug.

Both liposomal and particulate immunosuppressive agent dosage forms can be prepared using known techniques therefor. Liposome encapsulation of compounds is known in the art and discussed, for example, in U.S. Patent Nos. 4,948,590, 5,047,245 and 4,885,172. The preparation of particulate dosage

forms is discussed with respect to particulate clearing agent dosage forms in Example IX.

Cyclosporin A, for example, has a blood clearance half-life of about 6 hours. The major toxicities of cyclosporin A are renal, and nephrotoxicity occurs in 25-75% of patients treated with the drug. This toxicity frequently mandates cessation or modification of cyclosporin A treatment. A treatment regimen of cyclosporin A is also costly. A liposomal formulation of cyclosporin A is expected to show an increased serum residence time, increased bioavailability, and decreased toxicity in comparison to the free drug. Such liposomal dosage forms would therefore exhibit an increase in therapeutic index over that attainable through administration of free immunosuppressive drug compositions. Increased bioavailability should result in a decrease in the amount of drug and/or drug administrations necessary for efficacious treatment, thereby decreasing overall treatment cost.

Non-specific pretargeting techniques are also contemplated by the present invention. Such pretargeting techniques contemplate administration of a non-specific targeting moiety-ligand or a non-specific targeting moiety-anti-ligand conjugate. The non-specific targeting moiety equilibrates between the extravascular and vascular physiological compartment. Non-specific targeting moiety-containing conjugate becomes trapped in the extravascular compartment and is cleared from the vascular compartment by the recipient's excretory mechanisms or via the use of a clearing agent or clearing mechanism in accordance with the present invention.

Preferably, the non-specific targeting moiety-containing conjugate is administered, equilibrates between the extravascular compartment and the vascular compartment, and the conjugate in the vascular space is cleared using a clearing agent or a clearing

mechanism. Active agent-anti-ligand or active agent-ligand conjugate is then administered and such active agent-containing conjugate accretes to the extravascular compartment localized non-specific targeting moiety-containing conjugate and becomes associated therewith via ligand-anti-ligand interaction. The active agent is maintained in the extravascular space for a time sufficient to exert a therapeutic benefit at the target site.

Non-specific pretargeting allows the use of large, non-immunogenic proteins (e.g., IgG and IgM) for radioimmunotherapy, for example. Irrelevant antibodies that are non-immunogenic or less immunogenic than their specific antibody counterparts, and other non-specific targeting moieties may also be used in accordance with this aspect of the present invention. These targeting moiety-containing conjugates (non-specific targeting moiety-ligand or non-specific targeting moiety-anti-ligand) may be administered in large doses, greater than the dose administered using specific murine antibody-containing conjugates. A clearing agent or clearing mechanism is preferably administered or conducted between from about 18 to about 48 hours following non-specific targeting moiety containing conjugate administration. Active agent-anti-ligand or active agent-ligand is administered between from about 2 to about 6 hours after the clearing agent administration (or between about 18 to 120 hours following the non-specific targeting moiety-containing conjugate if a clearing agent or mechanism is not used).

Short half-life active agents, such as alpha-emitters, rapidly degraded active agents and the like, exert a therapeutic effect within a short time frame. Such active agents are therefore particularly amenable to use in non-specific pretargeting protocols, because the conjugate need not reside in the extravascular

space for an extended amount of time to deliver a therapeutically effective dose to target sites.

Example XXII describes experimentation involving a non-specific antibody-streptavidin conjugate; clearing agent; Y-90 chelate-labeled biotin pretargeting protocol that produced anti-tumor effects in mice, apparently through non-specific localization into the tumor interstitial fluid located in the extravascular compartment. Such protocols are useful for other diagnostic or therapeutic purposes for target sites that are located in a physiological compartment other than the vasculature. Inflammation imaging and the like are amenable to non-specific pretargeting diagnostic or therapeutic protocols.

Different immunogenicity reducing measures may be employed in tandem. That is, one conjugate component could be rendered less immunogenic using one chemical modification procedure, and an immunosuppressive agent could be employed to render the chemically modified conjugate less immunogenic. For example, the targeting moiety could be rendered less immunogenic via charge modification with succinic anhydride, and cyclosporin A could be administered both before and after administration of the succinylated conjugate. Other combinations of immunogenicity-reducing measures are also contemplated by the present invention.

A preferred targeting moiety useful in these embodiments of the present invention is a monoclonal antibody. Protein-protein conjugations are generally problematic due to the formation of undesirable byproducts, including high molecular weight and cross-linked species, however. A non-covalent synthesis technique involving reaction of biotinylated antibody with streptavidin has been reported to result in substantial byproduct formation. Also, at least one of the four biotin binding sites on the streptavidin is used to link the antibody and streptavidin, while

another such binding site may be sterically unavailable for biotin binding due to the configuration of the streptavidin-antibody conjugate.

Thus, covalent streptavidin-antibody conjugation is preferred, but high molecular weight byproducts are often obtained. The degree of crosslinking and aggregate formation is dependent upon several factors, including the level of protein derivitization using heterobifunctional crosslinking reagents. Sheldon et al., Appl. Radiat. Isot. 43: 1399-1402, 1992, discuss preparation of covalent thioether conjugates by reacting succinimidyl 4-(N-maleimidomethyl)cyclohexane -1-carboxylate (SMCC)-derivatized antibody and iminothiolane-derivatized streptavidin.

Streptavidin-proteinaceous targeting moiety conjugates are preferably prepared as described in Example XI below, with the preparation involving the steps of: preparation of SMCC-derivatized streptavidin; preparation of DTT-reduced proteinaceous targeting moiety; conjugation of the two prepared moieties; and purification of the monosubstituted or disubstituted (with respect to streptavidin) conjugate from crosslinked (antibody-streptavidin-antibody) and aggregate species and unreacted starting materials. The purified fraction is preferably further characterized by one or more of the following techniques: HPLC size exclusion, SDS-PAGE, immunoreactivity, biotin binding capacity and in vivo studies.

Alternatively, thioether conjugates useful in the practice of the present invention may be formed using other thiolating agents, such as SPDP, iminothiolane, SATA or the like, or other thio-reactive heterobifunctional cross linkers, such as m-maleimidobenzoyl-N-hydroxysuccinimide ester, N-succinimidyl(4-iodoacetyl)aminobenzoate or the like.

Streptavidin-proteinaceous targeting moiety conjugates of the present invention can also be formed by conjugation of a lysine epsilon amino group of one protein with a maleimide-derivatized form of the other protein. For example, at pH 8-10, lysine epsilon amino moieties react with protein maleimides, prepared, for instance, by treatment of the protein with SMCC, to generate stable amine covalent conjugates. In addition, conjugates can be prepared by reaction of lysine epsilon amino moieties of one protein with aldehyde functionalities of the other protein. The resultant imine bond is reducible to generate the corresponding stable amine bond. Aldehyde functionalities may be generated, for example, by oxidation of protein sugar residues or by reaction with aldehyde-containing heterobifunctional cross linkers.

Another method of forming streptavidin-targeting moiety conjugates involves immobilized iminobiotin that binds SMCC-derivatized streptavidin. In this conjugation/purification method, the reversible binding character of iminobiotin (immobilized) to streptavidin is exploited to readily separate conjugate from the unreacted targeting moiety. Iminobiotin binding can be reversed under conditions of lower pH and elevated ionic strength, e.g., NH_2OAc, pH 4 (50 mM) with 0.5 M NaCl.

For streptavidin, for example, the conjugation/purification proceeds as follows:

- SMCC-derivatized streptavidin is bound to immobilized iminobiotin (Pierce Chemical Co., St. Louis, Missouri), preferably in column format;

- a molar excess (with respect to streptavidin) of DTT-reduced antibody (preferably free of reductant) is added to the nitrogen-purged, phosphate-buffered iminobiotin column wherein the SMCC-streptavidin is bound (DTT-reduced antibody will saturate the bound

SMCC-streptavidin, and unbound reduced antibody passing through the column can be reused);

- the column is washed free of excess antibody; and

- a buffer that lowers the pH and increases ionic strength is added to the column to elute streptavidin-antibody conjugate in pure form.

As indicated above, targeting moiety-mediated ligand-anti-ligand pretargeting involves the localization of either targeting moiety-ligand or targeting moiety-anti-ligand at target tissue. Often, peak uptake to such target tissue is achieved before the circulating level of targeting moiety-containing conjugate in the blood is sufficiently low to permit the attainment of an optimal target-to-non-target conjugate ratio. To obviate this problem, two approaches are useful. The first approach allows the targeting moiety-containing conjugate to clear from the blood by "natural" or endogenous clearance mechanisms. This method is complicated by variations in systemic clearance of proteins and by endogenous ligand or anti-ligand. For example, endogenous biotin may interfere with the preservation of biotin binding sites on a streptavidin-targeting moiety conjugate.

The second approach for improving targeting moiety-ligand or targeting moiety-anti-ligand conjugate target-to-blood ratio "chases" the conjugate from the circulation through in vivo complexation of conjugate with a molecule constituting or containing the complementary anti-ligand or ligand. When biotinylated antibodies are used as a ligand-targeting moiety conjugate, for example, avidin forms relatively large aggregated species upon complexation with the circulating biotinylated antibody, which aggregated species are rapidly cleared from the blood by the RES uptake. See, for example, U.S. Patent No. 4,863,713. One problem with this method, however, is the

potential for cross-linking and internalizing tumor-bound biotinylated antibody by avidin.

When avidin-targeting moiety conjugates are employed, poly-biotinylated transferrin has been used to form relatively large aggregated species that are cleared by RES uptake. See, for example, Goodwin, J. Nucl. Med. 33(10):1816-18, 1992). Poly-biotinylated transferrin also has the potential for cross-linking and internalizing tumor-bound avidinylated-targeting moiety, however. In addition, both "chase" methodologies involve the prolonged presence of aggregated moieties of intermediate, rather than large, size (which are not cleared as quickly as large size particles by RES uptake), thereby resulting in serum retention of subsequently administered ligand-active agent or anti-ligand-active agent. Such serum retention unfavorably impacts the target cell-to-blood targeting ratio.

The present invention provides clearing agents of protein and non-protein composition having physical properties facilitating use for in vivo complexation and blood clearance of anti-ligand/ligand (e.g., avidin/biotin)-targeting moiety (e.g., antibody) conjugates. These clearing agents are useful in improving the target:blood ratio of targeting moiety conjugate. Other applications of these clearing agents include lesional imaging or therapy involving blood clots and the like, employing antibody-active agent delivery modalities. For example, efficacious anti-clotting agent provides rapid target localization and high target:non-target targeting ratio. Active agents administered in pretargeting protocols of the present invention using efficient clearing agents are targeted in the desirable manner and are, therefore, useful in the imaging/therapy of conditions such as pulmonary embolism and deep vein thrombosis.

Clearing agents useful in the practice of the present invention preferably exhibit one or more of the following characteristics:

- rapid, efficient complexation with targeting moiety-ligand (or anti-ligand) conjugate in vivo;
- rapid clearance from the blood of targeting moiety conjugate capable of binding a subsequently administered complementary anti-ligand or ligand containing molecule;
- high capacity for clearing (or inactivating) large amounts of targeting moiety conjugate; and
- low immunogenicity.

Preferred clearing agents include hexose-based and non-hexose based moieties. Hexose-based clearing agents are molecules that have been derivatized to incorporate one or more hexoses (six carbon sugar moieties) recognized by Ashwell receptors or other receptors such as the mannose/N-acetylglucosamine receptor which are associated with endothelial cells and/or Kupffer cells of the liver or the mannose 6-phosphate receptor. Exemplary of such hexoses are galactose, mannose, mannose 6-phosphate, N-acetylglusosamine and the like. Other moieties recognized by Ashwell receptors, including glucose, N-galactosamine, N-acetylgalactosamine, thioglycosides of galactose and, generally, D-galactosides and glucosides or the like may also be used in the practice of the present invention. Galactose is the prototypical clearing agent hexose derivative for the purposes of this description. Galactose thioglycoside conjugation to a protein is preferably accomplished in accordance with the teachings of Lee et al., "2-Imino-2-methoxyethyl 1-Thioglycosides: New Reagents for Attaching Sugars to Proteins," Biochemistry, 15(18): 3956, 1976. Another useful galactose thioglycoside conjugation method is set forth in Drantz et al, "Attachment of Thioglycosides to Proteins:

Enhancement of Liver Membrane Binding," Biochemistry, 15(18): 3963, 1976. Thus, galactose-based and non-galactose based molecules are discussed below.

Protein-type galactose-based clearing agents include proteins having endogenous exposed galactose residues or which have been derivatized to expose or incorporate such galactose residues. Exposed galactose residues direct the clearing agent to rapid clearance by endocytosis into the liver through specific receptors therefor (Ashwell receptors). These receptors bind the clearing agent, and induce endocytosis into the hepatocyte, leading to fusion with a lysosome and recycle of the receptor back to the cell surface. This clearance mechanism is characterized by high efficiency, high capacity and rapid kinetics.

An exemplary clearing agent of the protein-based/galactose-bearing variety is the asialoorosomucoid derivative of human alpha-1 acid glycoprotein (orosomucoid, molecular weight = 41,000 Dal, isoelectric point = 1.8-2.7). The rapid clearance from the blood of asialoorosomucoid has been documented by Galli, et al., J. of Nucl. Med. Allied Sci. 32(2): 110-16, 1988.

Treatment of orosomucoid with neuraminidase removes sialic acid residues, thereby exposing galactose residues. Other such derivatized clearing agents include, for example, galactosylated albumin, galactosylated-IgM, galactosylated-IgG, asialohaptoglobin, asialofetuin, asialoceruloplasmin and the like.

Human serum albumin (HSA), for example, may be employed in a clearing agent of the present invention as follows:

$(Hexose)_m$--Human Serum Albumin (HSA)--$(Ligand)_n$,

wherein n is an integer from 1 to about 10 and m is an integer from 1 to about 25 and wherein the hexose is recognized by Ashwell receptors.

In a preferred embodiment of the present invention the ligand is biotin and the hexose is galactose. More preferably, HSA is derivatized with from 10-20 galactose residues and 1-5 biotin residues. Still more preferably, HSA clearing agents of the present invention are derivatized with from about 12 to about 15 galactoses and 3 biotins. Derivatization with both galactose and biotin are conducted in a manner sufficient to produce individual clearing agent molecules with a range of biotinylation levels that averages a recited whole number, such as 1, biotin. Derivatization with 3 biotins, for example, produces a product mixture made up of individual clearing agent molecules, substantially all of which having at least one biotin residue. Derivatization with 1 biotin produces a clearing agent product mixture, wherein a significant portion of the individual molecules are not biotin derivatized. The whole numbers used in this description refer to the average biotinylation of the clearing agents under discussion.

In addition, clearing agents based upon human proteins, especially human serum proteins, such as, for example, orosomucoid and human serum albumin, human IgG, human-anti-antibodies of IgG and IgM class and the like, are less immunogenic upon administration into the serum of a human recipient. Another advantage of using asialoorosomucoid is that human orosomucoid is commercially available from, for example, Sigma Chemical Co, St. Louis, Missouri.

One way to prevent clearing agent compromise of target-bound conjugate through direct complexation is through use of a clearing agent of a size sufficient to render the clearing agent less capable of diffusion into the extravascular space and binding to target-

associated conjugate. This strategy is useful alone or in combination with the aforementioned recognition that exposed galactose residues direct rapid liver uptake. This size-exclusion strategy enhances the effectiveness of non-galactose-based clearing agents of the present invention. The combination (exposed galactose and size) strategy improves the effectiveness of "protein-type" or "polymer-type" galactose-based clearing agents.

Galactose-based clearing agents include galactosylated, biotinylated proteins (to remove circulating streptavidin-targeting moiety conjugates, for example) of intermediate molecular weight (ranging from about 40,000 to about 200,000 Dal), such as biotinylated asialoorosomucoid, galactosyl-biotinyl-human serum albumin or other galactosylated and biotinylated derivatives of non-immunogenic soluble natural proteins, as well as biotin- and galactose-derivatized polyglutamate, polylysine, polyarginine, polyaspartate and the like. High molecular weight moieties (ranging from about 200,000 to about 1,000,000 Dal) characterized by poor target access, including galactosyl-biotinyl-IgM or -IgG (approximately 150,000 Dal) molecules, as well as galactose- and biotin-derivatized transferrin conjugates of human serum albumin, IgG and IgM molecules and the like, can also be used as clearing agents of the claimed invention. Chemically modified polymers of intermediate or high molecular weight (ranging from about 40,000 to about 1,000,000 Dal), such as galactose- and biotin-derivatized dextran, hydroxypropylmethacrylamide polymers, polyvinylpyrrolidone-polystyrene copolymers, divinyl ether-maleic acid copolymers, pyran copolymers, or PEG, also have utility as clearing agents in the practice of the present invention. In addition, rapidly clearing biotinylated liposomes (high

molecular weight moieties with poor target access) can be derivatized with galactose and biotin to produce clearing agents for use in the practice of the present invention.

A further class of clearing agents useful in the present invention involve small molecules (ranging from about 500 to about 10,000 Dal) derivatized with galactose and biotin that are sufficiently polar to be confined to the vascular space as an in vivo volume of distribution. More specifically, these agents exhibit a highly charged structure and, as a result, are not readily distributed into the extravascular volume, because they do not readily diffuse across the lipid membranes lining the vasculature. Exemplary of such clearing agents are mono- or poly-biotin-derivatized 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo) bis[4-amino-5-hydroxy-1,3-naphthalene disulfonic acid] tetrasodium salt, mono- or poly-biotinyl-galactose-derivatized polysulfated dextran-biotin, mono- or poly-biotinyl-galactose-derivatized dextran-biotin and the like.

The galactose-exposed or -derivatized clearing agents are preferably capable of (1) rapidly and efficiently complexing with the relevant ligand- or anti-ligand-containing conjugates via ligand-anti-ligand affinity; and (2) clearing such complexes from the blood via the galactose receptor, a liver specific degradation system, as opposed to aggregating into complexes that are taken up by the generalized RES system, including the lung and spleen. Additionally, the rapid kinetics of galactose-mediated liver uptake, coupled with the affinity of the ligand-anti-ligand interaction, allow the use of intermediate or even low molecular weight carriers.

Non-galactose residue-bearing moieties of low or intermediate molecular weight (ranging from about 40,000 to about 200,000 Dal) localized in the blood

may equilibrate with the extravascular space and, therefore, bind directly to target-associated conjugate, compromising target localization. In addition, aggregation-mediated clearance mechanisms operating through the RES system are accomplished using a large stoichiometric excess of clearing agent. In contrast, the rapid blood clearance of galactose-based clearing agents used in the present invention prevents equilibration, and the high affinity ligand-anti-ligand binding allows the use of low stoichiometric amounts of such galactose-based clearing agents. This feature further diminishes the potential for galactose-based clearing agents to compromise target-associated conjugate, because the absolute amount of such clearing agent administered is decreased.

Clearing agent evaluation experimentation involving galactose- and biotin-derivatized clearing agents of the present invention is detailed in Examples XIII and XVII. Specific clearing agents of the present invention that were examined during the Example XVII experimentation are (1) asialoorosomucoid-biotin, (2) human serum albumin derivatized with galactose and biotin, and (3) a 70,000 dalton molecular weight dextran derivatized with both biotin and galactose. The experimentation showed that proteins and polymers are derivatizable to contain both galactose and biotin and that the resultant derivatized molecule is effective in removing circulating streptavidin-protein conjugate from the serum of the recipient. Biotin loading was varied to determine the effects on both clearing the blood pool of circulating avidin-containing conjugate and the ability to deliver a subsequently administered biotinylated isotope to a target site recognized by the streptavidin-containing conjugate. The effect of

relative doses of the administered components with respect to clearing agent efficacy was also examined.

Protein-type and polymer-type non-galactose-based clearing agents include the agents described above, absent galactose exposure or derivitization and the like. These clearing agents act through an aggregation-mediated RES mechanism. In these embodiments of the present invention, the clearing agent used will be selected on the basis of the target organ to which access of the clearing agent is to be excluded. For example, high molecular weight (ranging from about 200,000 to about 1,000,000 Dal) clearing agents will be used when tumor targets or clot targets are involved.

Another class of clearing agents includes agents that do not remove circulating ligand or anti-ligand/targeting moiety conjugates, but instead "inactivate" the circulating conjugates by blocking the relevant anti-ligand or ligand binding sites thereon. These "cap-type" clearing agents are preferably small (500 to 10,000 Dal) highly charged molecules, which exhibit physical characteristics that dictate a volume of distribution equal to that of the plasma compartment (i.e., do not extravasate into the extravascular fluid volume). Exemplary cap-type clearing agents are poly-biotin-derivatized 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo) bis[4-amino-5-hydroxy-1,3-naphthalene disulfonic acid] tetrasodium salt, poly-biotinyl-derivatized polysulfated dextran-biotin, mono- or poly-biotinyl-derivatized dextran-biotin and the like.

Cap-type clearing agents are derivatized with the relevant anti-ligand or ligand, and then administered to a recipient of previously administered ligand/ or anti-ligand/targeting moiety conjugate. Clearing agent-conjugate binding therefore diminishes the ability of circulating conjugate to bind any

subsequently administered active agent-ligand or active agent-anti-ligand conjugate. The ablation of active agent binding capacity of the circulating conjugate increases the efficiency of active agent delivery to the target, and increases the ratio of target-bound active agent to circulating active agent by preventing the coupling of long-circulating serum protein kinetics with the active agent. Also, confinement of the clearing agent to the plasma compartment prevents compromise of target-associated ligand or anti-ligand.

Clearing agents of the present invention may be administered in single or multiple doses. A single dose of biotinylated clearing agent, for example, produces a rapid decrease in the level of circulating targeting moiety-streptavidin, followed by a small increase in that level, presumably caused, at least in part, by re-equilibration of targeting moiety-streptavidin within the recipient's physiological compartments. A second or additional clearing agent doses may then be employed to provide supplemental clearance of targeting moiety-streptavidin. Alternatively, clearing agent may be infused intravenously for a time period sufficient to clear targeting moiety-streptavidin in a continuous manner.

Other types of clearing agents and clearance systems are also useful in the practice of the present invention to remove circulating targeting moiety-ligand or -anti-ligand conjugate from the recipient's circulation. Particulate-based clearing agents, for example, are discussed in Example IX. In addition, extracorporeal clearance systems are discussed in Example IX. In vivo clearance protocols employing arterially inserted proteinaceous or polymeric multiloop devices are also described in Example IX.

For monoclonal antibody-streptavidin conjugates, for example, the targeting moiety portion of the

conjugate may be humanized or may constitute a fully human antibody to address the immunogenicity issue. Such techniques cannot be employed with streptavidin, a bacterial protein. The effect of the clearing agent of the present invention on streptavidin immunogenicity was studied. These studies, discussed in Example XVIII, featured multiple streptavidin-containing conjugate administration and showed that mice that also received clearing agent exhibited lower immune responses to streptavidin than did mice that did not receive clearing agent. Consequently, clearing agents in accordance with the present invention that direct the excretion of streptavidin-containing conjugate also exhibit utility as immunosuppressive agents. The amount of clearing agent used in these experiments had been previously shown to be effective with respect to serum clearance of the streptavidin-containing conjugate.

There are several possible explanations for the ability of the clearing agent to attenuate streptavidin immunogenicity. One such possibility is that the streptavidin-containing conjugate is simply cleared from the recipient too quickly for anti-streptavidin antibody formation to occur. In this case, any method of rapid serum clearance, such as particulate clearing agents or extracorporeal clearance methods or the like, would be expected to function similarly.

Another possible explanation for the immunosuppressive effect of clearing agent is that the streptavidin-containing conjugate is rapidly cleared from the circulation and quickly metabolically degraded prior to antigen presentation to the immune system. Such metabolic degradation would presumably generate moieties that are no longer recognized as non-self. Alternatively, the clearance mechanism utilized by the clearing agent, e.g., an Ashwell

receptor mechanism, delivers the streptavidin-containing conjugate to a physiological compartment that is less readily exposed to the immune system. In these latter cases, any clearing agent that operates using such mechanisms would be expected to attenuate streptavidin-containing conjugate immunogenicity.

One embodiment of the present invention in which rapid acting clearing agents are useful is in the delivery of Auger emitters, such as I-125, I-123, Er-165, Sb-119, Hg-197, Ru-97, Tl-201 and I-125 and Br-77, or nucleus-binding drugs to target cell nuclei. In these embodiments of the present invention, targeting moieties that localize to internalizing receptors on target cell surfaces are employed to deliver a targeting moiety-containing conjugate (i.e., a targeting moiety-anti-ligand conjugate in the preferred two-step protocol) to the target cell population. Such internalizing receptors include EGF receptors, transferrin receptors, HER2 receptors, IL-2 receptors, other interleukins and cluster differentiation receptors, somatostatin receptors, other peptide binding receptors and the like.

After the passage of a time period sufficient to achieve localization of the conjugate to target cells, but insufficient to induce internalization of such targeted conjugates by those cells through a receptor-mediated event, a rapidly acting clearing agent is administered. In a preferred two-step protocol, an active agent-containing ligand or anti-ligand conjugate, such as a biotin-Auger emitter or a biotin-nucleus acting drug, is administered as soon as the clearing agent has been given an opportunity to complex with circulating targeting moiety-containing conjugate, with the time lag between clearing agent and active agent administration being less than about 24 hours. In this manner, active agent is readily internalized through target cell receptor-mediated

internalization. While circulating Auger emitters are thought to be non-toxic, the rapid, specific targeting afforded by the pretargeting protocols of the present invention increases the potential of shorter half-life Auger emitters, such as I-123, which is available and capable of stable binding.

In order to more effectively deliver a therapeutic or diagnostic dose of radiation to a target site, the radionuclide is preferably retained at the tumor cell surface. Loss of targeted radiation occurs as a consequence of metabolic degradation mediated by metabolically active target cell types, such as tumor or liver cells.

Preferable agents and protocols within the present invention are therefore characterized by prolonged residence of radionuclide at the target cell site to which the radionuclide has localized and improved radiation absorbed dose deposition at that target cell site, with decreased targeted radioactivity loss resulting from metabolism. Radionuclides that are particularly amenable to the practice of this aspect of the present invention are rhenium, iodine and like "non +3 charged" radiometals which exist in chemical forms that easily cross cell membranes and are not, therefore, inherently retained by cells. In contrast, radionuclides having a +3 charge, such as In-111, Y-90, Lu-177 and Ga-67, exhibit natural target cell retention as a result of their containment in high charge density chelates.

Evidence exists that streptavidin is resistant to metabolic degradation. Consequently, radionuclide bound directly or indirectly to streptavidin, rather than, for example, directly to the targeting moiety, are retained at target cell sites for extended periods of time, as described below in Examples XIV and XV. Streptavidin-associated radionuclide can be

administered in pretargeting protocols or injected directly into lesions.

In addition, streptavidin-associated radionuclide (e.g., streptavidin-radionuclide and streptavidin-biotin-radionuclide) may be administered as such (in pretargeting protocols) or as conjugates incorporating targeting moieties (intralesional injection and pretargeting protocols) specific for stable target cell surface antigens (such as NR-LU-10 antibody, L6, anti-CEA antibodies or the like) or target cell internalizing antigens (such as anti-HER2^{neu}; anti-epidermal growth factor; anti-Lewis Y, including B-1, B-3, BR-64, BR-96 and the like; or the like) to target the streptavidin to the appropriate target cell population.

Streptavidin associated-radionuclides are amenable, for example, to intralesional injection of ovarian cancer lesions studded on the peritoneum and accessible via laparotomy. Another example of an intralesional injection aspect of the present invention involves hepatoma or liver cancer, preferably using a terminal galactose-streptavidin derivative to bind a radionuclide.

Moreover, high molecular weight carriers, such as biodegradable particles, dextran, albumin or the like, may be employed (e.g., conjugated to streptavidin) to limit leakage of the administered streptavidin from the injection site. Alternatively, such carriers are biotinylated, thereby constituting suitable targets or carriers for radionuclide-streptavidin molecules.

The use of streptavidin-associated radionuclide in intralesional injection protocols provides the following advantages:

- less radionuclide is used to better advantage, because the therapeutic efficacy of the administered radionuclide is improved as a result of retention at the target cell site;

- microdiffusion from the injection site results in expansion of the field of radiation deposition;

- minimized toxicity and higher dose rate radiation are achieved;

- combination with modalities exhibiting disparate toxicity profiles may be useful;

- target sites are imageable post-injection to allow dosimetry determinations to be made;

- biodegradable (i.e., not requiring removal) retention moiety-carrier molecules can be utilized; and

- repeated doses can be injected, because local administration without systemic distribution minimizes antiglobulin response.

The use of streptavidin-associated radionuclide in pretargeting protocols provides the following advantages:

- less radionuclide is used to better advantage, because the therapeutic efficacy of the administered radionuclide is improved as a result of retention at the target cell site;

- target sites are imageable post-injection to allow dosimetry determinations to be made;

- minimized toxicity and higher dose rate radiation are achieved; and

- combination with modalities exhibiting disparate toxicity profiles may be useful.

In addition, the target cell retention-enhancing aspect of the present invention is applicable to a hybrid pretargeting/intralesional injection protocol. For example, targeting moiety-biotin conjugate is administered and an intralesional injection of streptavidin follows after a time sufficient to permit localization of the targeting moiety-biotin conjugate to target cell sites of reasonably determinable location. Next, a radionuclide-biotin molecule is administered, wherein this administration is conducted

by intralesional, intravenous or other convenient route.

Monovalent antibody fragment-streptavidin conjugate may be used to pretarget streptavidin, preferably in additional embodiments of the two-step aspect of the present invention. Exemplary monovalent antibody fragments useful in these embodiments are Fv, Fab, Fab' and the like. Monovalent antibody fragments, typically exhibiting a molecular weight ranging from about 25 kD (Fv) to about 50 kD (Fab, Fab'), are smaller than whole antibody and, therefore, are generally capable of greater target site penetration. Moreover, monovalent binding can result in less binding carrier restriction at the target surface (occurring during use of bivalent antibodies, which bind strongly and adhere to target cell sites thereby creating a barrier to further egress into sublayers of target tissue), thereby improving the homogeneity of targeting.

In addition, smaller molecules are more rapidly cleared from a recipient, thereby decreasing the immunogenicity of the administered small molecule conjugate. A lower percentage of the administered dose of a monovalent fragment conjugate localizes to target in comparison to a whole antibody conjugate. The decreased immunogenicity may permit a greater initial dose of the monovalent fragment conjugate to be administered, however.

A multivalent, with respect to ligand, moiety is preferably then administered. This moiety also has one or more radionuclide associated therewith. As a result, the multivalent moiety serves as both a clearing agent for circulating anti-ligand-containing conjugate (through cross-linking or aggregation of conjugate) and as a therapeutic agent when associated with target bound conjugate. In contrast to the internalization caused by cross-linking described

above, cross-linking at the tumor cell surface stabilizes the monovalent fragment-anti-ligand molecule and, therefore, enhances target retention, under appropriate conditions of antigen density at the target cell. In addition, monovalent antibody fragments generally do not internalize as do bivalent or whole antibodies. The difficulty in internalizing monovalent antibodies permits cross-linking by a monovalent moiety serves to stabilize the bound monovalent antibody through multipoint binding. This two-step protocol of the present invention has greater flexibility with respect to dosing, because the decreased fragment immunogenicity allows more streptavidin-containing conjugate, for example, to be administered, and the simultaneous clearance and therapeutic delivery removes the necessity of a separate controlled clearing step.

Another embodiment of the pretargeting methodologies of the present invention involves the route of administration of the ligand- or anti-ligand-active agents. In these embodiments of the present invention, the active agent-ligand (e.g., radiolabeled biotin) or -anti-ligand is administered intraarterially using an artery supplying tissue that contains the target. In the radiolabeled biotin example, the high extraction efficiency provided by avidin-biotin interaction facilitates delivery of very high radioactivity levels to the target cells, provided the radioactivity specific activity levels are high. The limit to the amount of radioactivity delivered therefore becomes the biotin binding capacity at the target (i.e., the amount of antibody at the target and the avidin equivalent attached thereto).

For these embodiments of the pretargeting methods of the present invention, particle emitting therapeutic radionuclide resulting from transmutation

processes (without non-radioactive carrier forms present) are preferred. Exemplary radionuclides include Y-90, Re-188, At-211, Bi-212 and the like. Other reactor-produced radionuclides are useful in the practice of these embodiments of the present invention, if they are able to bind in amounts delivering a therapeutically effective amount of radiation to the target. A therapeutically effective amount of radiation ranges from about 1500 to about 10,000 cGy depending upon several factors known to nuclear medicine practitioners.

Intraarterial administration pretargeting can be applied to targets present in organs or tissues for which supply arteries are accessible. Exemplary applications for intraarterial delivery aspects of the pretargeting methods of the present invention include treatment of liver tumors through hepatic artery administration, brain primary tumors and metastases through carotid artery administration, lung carcinomas through bronchial artery administration and kidney carcinomas through renal artery administration. Intraarterial administration pretargeting can be conducted using chemotherapeutic drug, toxin and anti-tumor active agents as discussed below. High potency drugs, lymphokines, such as IL-2 and tumor necrosis factor, drug/lymphokine-carrier-biotin molecules, biotinylated drugs/lymphokines, and drug/lymphokine/toxin-loaded, biotin-derivatized liposomes are exemplary of active agents and/or dosage forms useful for the delivery thereof in the practice of this embodiment of the present invention.

Route of administration also impacts the immunogenicity of administered molecules. For monoclonal antibody-streptavidin conjugate, for example, experiments have shown that intravenous administration renders the conjugate less immunogenic than intramuscular administration, for example. In

addition, local administration, such as the intraarterial administration discussed above and direct intralesional administration, of conjugate generally renders that conjugate less immunogenic. Other examples include intraperitoneal administration for ovarian cancer, intralymphatic administration for lymph node metastases secondary to cervical cancer, intrapleural administration for intractable pleural effusion, and intrapericardially for pericardial effusion and impending cardiac tamponade.

In embodiments of the present invention employing radionuclide therapeutic agents, the rapid clearance of nontargeted therapeutic agent decreases the exposure of non-target organs, such as bone marrow, to the therapeutic agent. Consequently, higher doses of radiation can be administered absent dose limiting bone marrow toxicity. In addition, pretargeting methods of the present invention optionally include administration of short duration bone marrow protecting agents, such as WR 2721. As a result, even higher doses of radiation can be given, absent dose limiting bone marrow toxicity.

While the pretargeting protocols set forth above have been described primarily in combination with delivery of a radionuclide diagnostic or therapeutic moiety, the protocols are amenable to use for delivery of other moieties, including anti-tumor agents, chemotherapeutic drugs and the like. For example, most naturally occurring and recombinant cytokines have short in vivo half lives. This characteristic limits the clinical effectiveness of these molecules, because near toxic doses are often required. Dose-limiting toxicities in humans have been observed upon high dose IL-2 or tumor necrosis factor administrations, for example.

A protocol, such as administration of streptavidin-targeting moiety conjugate followed by

administration of biotinylated cytokine, is also contemplated by the present invention. Such pretargeting of anti-ligand serves to improve the performance of cytokine therapeutics by increasing the amount of cytokine localized to target cells.

Streptavidin-antibody conjugates generally exhibit pharmacokinetics similar to the native antibody and localize well to target cells, depending upon their construction. Biotinylated cytokines retain a short in vivo half-life; however, cytokine may be localized to the target as a result of the affinity of biotin for avidin. In addition, biotin-avidin experience a pH-dependent dissociation which occurs at a slow rate, thereby permitting a relatively constant, sustained release of cytokine at the target site over time. Also, cytokines complexed to target cells through biotin-avidin association are available for extraction and internalization by cells involved in cellular-mediated cytotoxicity.

A pre-formed antibody-streptavidin-biotin-cytokine preparation may also be employed in the practice of these methods of the present invention. In addition, a three-step protocol of the present invention may also be employed to deliver a cytokine, such as IL-2, to a target site.

Other anti-tumor agents that may be delivered in accordance with the pretargeting techniques of the present invention are selectins, including L-selectin, P-selectin and E-selectin. The presence of cytokines stimulates cells, such as endothelial cells, to express selectins on the surfaces thereof. Selectins bind to white blood cells and aid in delivering white blood cells where they are needed. Consequently, a protocol, such as administration of streptavidin- or avidin-targeting moiety conjugate followed by administration of biotinylated selectins, is also contemplated by the present invention. Such

pretargeting of anti-ligand serves to improve the performance of selectin therapeutics by increasing the amount of selectin localized to target cells. In this manner, the necessity of cytokine induction of selectin expression is obviated by the localization and retention of selectin at a target cell population.

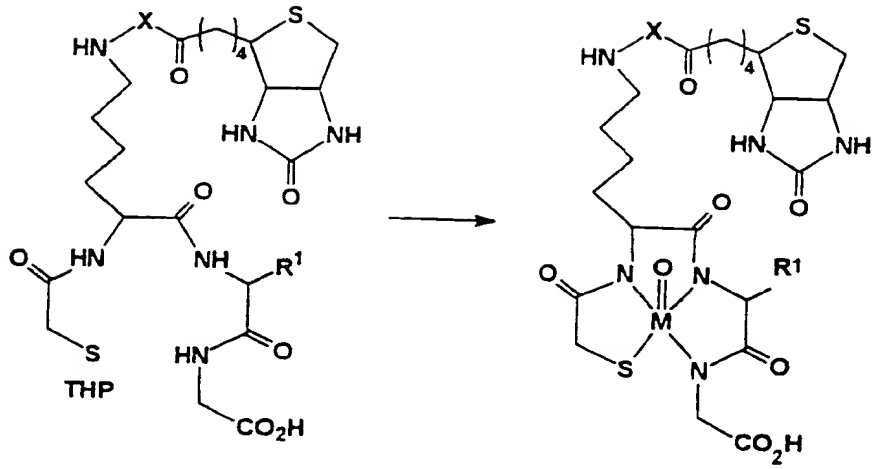
Chemotherapeutic drugs also generally exhibit short in vivo half-lives at a therapeutically effective dose. Consequently, another example of a protocol of the present invention includes administration of avidin-targeting moiety conjugate followed by administration of a biotin-chemotherapeutic drug conjugate or complex, such as a drug-carrier-biotin complex. A three-step protocol of the present invention may also be employed to deliver a chemotherapeutic drug, such as methotrexate, adriamycin, high potency adriamycin analogs, trichothecenes, potent enediynes, such as esperamycins and calicheamycins, cytoxan, vinca alkaloids, actinamycin D, taxol, taxotere or the like to a target site.

The invention is further described through presentation of the following examples. These examples are offered by way of illustration, and not by way of limitation.

Example I

Synthesis of a Chelate-Biotin Conjugate

A chelating compound that contains an N_3S chelating core was attached via an amide linkage to biotin. Radiometal labeling of an exemplary chelate-biotin conjugate is illustrated below.



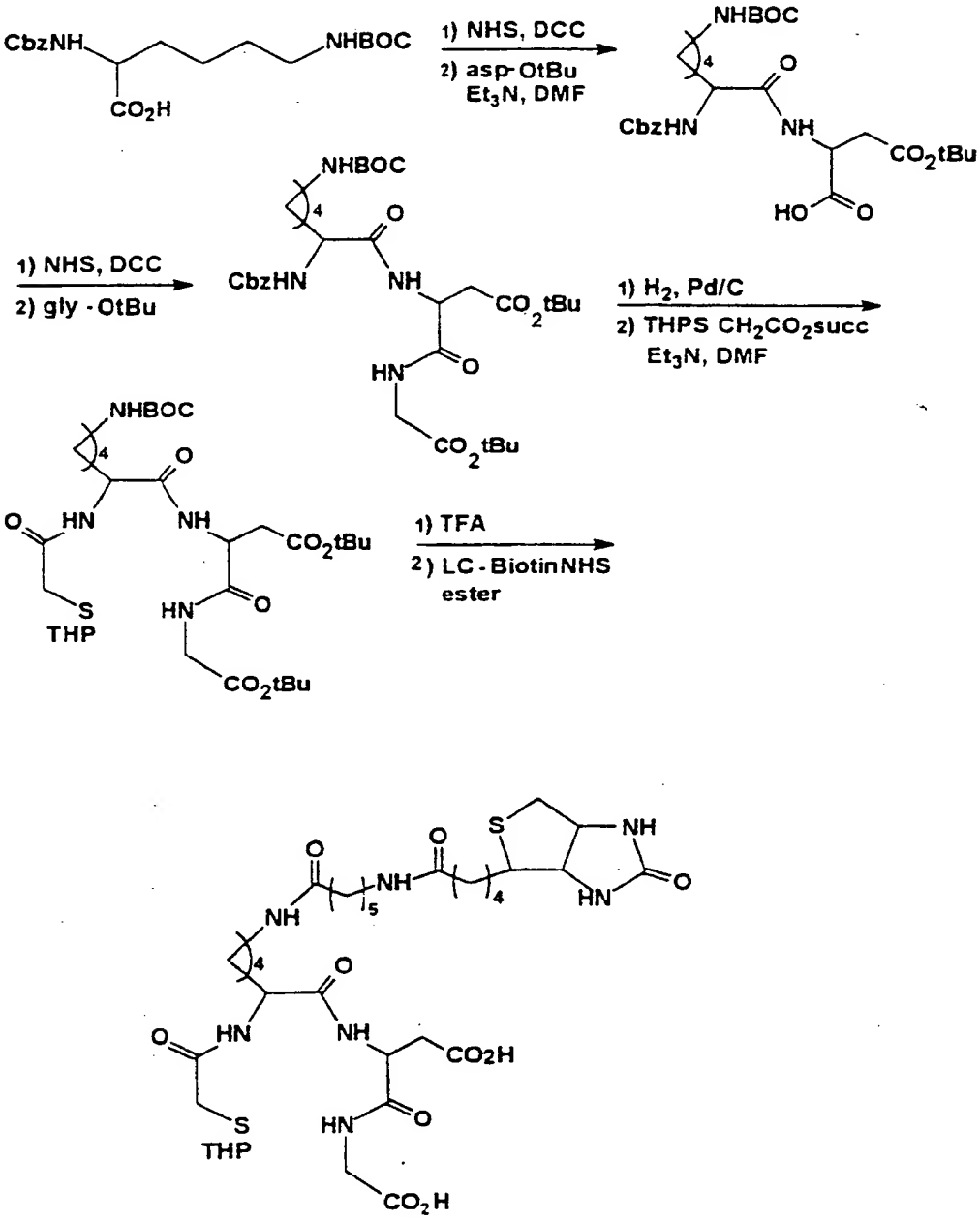
X = $(CH_2)_0$, short chain
X = $CO(CH_2)_5NH$, long chain
R^1 = H or CH_2CO_2H

M = Tc or Rc

The spacer group "X" permits the biotin portion of the conjugate to be sterically available for avidin binding. When "R^1" is a carboxylic acid substituent (for instance, CH_2COOH), the conjugate exhibits improved water solubility, and further directs in vivo excretion of the radiolabeled biotin conjugate toward renal rather than hepatobiliary clearance.

Briefly, N-α-Cbz-N-Σ-t-BOC protected lysine was converted to the succinimidyl ester with NHS and DCC, and then condensed with aspartic acid β--t-butyl ester. The resultant dipeptide was activated with NHS and DCC, and then condensed with glycine t-butyl ester. The Cbz group was removed by hydrogenolysis, and the amine was acylated using tetrahydropyranyl mercaptoacetic acid succinimidyl ester, yielding S-(tetrahydropyranyl)-mercaptoacetyl-lysine. Trifluoroacetic acid cleavage of the N-t-BOC group and t-butyl esters, followed by condensation with LC-

biotin-NHS ester provided (Σ-caproylamide biotin)-aspartyl glycine. This synthetic method is illustrated below.



^{1}H NMR: (CD_3OD, 200 MHz Varian): 1.25-1.95 (m, 24H), 2.15-2.25 (broad t, 4H), 2.65-3.05 (m, 4H), 3.30-3.45 (dd, 2H), 3.50-3.65 (ddd, 2H), 3.95 (broad s, 2H), 4.00-4.15 (m, 1H), 4.25-4.35 (m, 1H), 4.45-4.55 (m, 1H), 4.7-5.05 (m overlapping with HOD).

Elemental Analysis: C, H, N for $C_{35}H_{57}N_7O_{11}S_2 \cdot H_2O$

calculated: 50.41, 7.13, 11.76

found: 50.13, 7.14, 11.40

Example II

Preparation of a Technetium or Rhenium Radiolabeled Chelate-Biotin Conjugate

The chelate-biotin conjugate of Example I was radiolabeled with either ^{99m}Tc pertechnetate or ^{186}Re perrhenate. Briefly, ^{99m}Tc pertechnetate was reduced with stannous chloride in the presence of sodium gluconate to form an intermediate Tc-gluconate complex. The chelate-biotin conjugate of Example I was added and heated to 100°C for 10 min at a pH of about 1.8 to about 3.3. The solution was neutralized to a pH of about 6 to about 8, and yielded an N_3S-coordinated ^{99m}Tc-chelate-biotin conjugate. C-18 HPLC gradient elution using 5-60% acetonitrile in 1% acetic acid demonstrated two anomers at 97% or greater radiochemical yield using δ (gamma ray) detection.

Alternatively, ^{186}Re perrhenate was spiked with cold ammonium perrhenate, reduced with stannous chloride, and complexed with citrate. The chelate-biotin conjugate of Example I was added and heated to 90°C for 30 min at a pH of about 2 to 3. The solution was neutralized to a pH of about 6 to about 8, and yielded an N_3S-coordinated ^{186}Re-chelate-biotin conjugate. C-18 HPLC gradient elution using 5-60% acetonitrile in 1% acetic acid resulted in radiochemical yields of 85-90%. Subsequent purification over a C-18 reverse

phase hydrophobic column yielded material of 99% purity.

Example III

In Vitro Analysis of Radiolabeled Chelate-Biotin Conjugates

Both the ^{99m}Tc- and ^{186}Re-chelate-biotin conjugates were evaluated in vitro. When combined with excess avidin (about 100-fold molar excess), 100% of both radiolabeled biotin conjugates complexed with avidin.

A ^{99m}Tc-biotin conjugate was subjected to various chemical challenge conditions. Briefly, ^{99m}Tc-chelate-biotin conjugates were combined with avidin and passed over a 5 cm size exclusion gel filtration column. The radiolabeled biotin-avidin complexes were subjected to various chemical challenges (see Table 1), and the incubation mixtures were centrifuged through a size exclusion filter. The percent of radioactivity retained (indicating avidin-biotin-associated radiolabel) is presented in Table 1. Thus, upon chemical challenge, the radiometal remained associated with the macromolecular complex.

TABLE 1

Chemical Challenge of ^{99m}Tc-Chelate-Biotin-Avidin Complexes

Challenge Medium	pH	% Radioactivity Retained 1 h, 37°C	18 h, RT
PBS	7.2	99	99
Phosphate	8.0	97	97
10 mM cysteine	8.0	92	95
10 mM DTPA	8.0	99	98
0.2 M carbonate	10.0	97	94

In addition, each radiolabeled biotin conjugate was incubated at about 50 μg/ml with serum; upon completion of the incubation, the samples were subjected to instant thin layer chromatography (ITLC)

in 80% methanol. Only 2-4% of the radioactivity remained at the origin (i.e., associated with protein); this percentage was unaffected by the addition of exogenous biotin. When the samples were analyzed using size exclusion H-12 FPLC with 0.2 M phosphate as mobile phase, no association of radioactivity with serum macromolecules was observed.

Each radiolabeled biotin conjugate was further examined using a competitive biotin binding assay. Briefly, solutions containing varying ratios of D-biotin to radiolabeled biotin conjugate were combined with limiting avidin at a constant total biotin:avidin ratio. Avidin binding of each radiolabeled biotin conjugate was determined by ITLC, and was compared to the theoretical maximum stoichiometric binding (as determined by the HABA spectrophotometric assay of Green, Biochem. J. 94:23c-24c, 1965). No significant difference in avidin binding was observed between each radiolabeled biotin conjugate and D-biotin.

Example IV

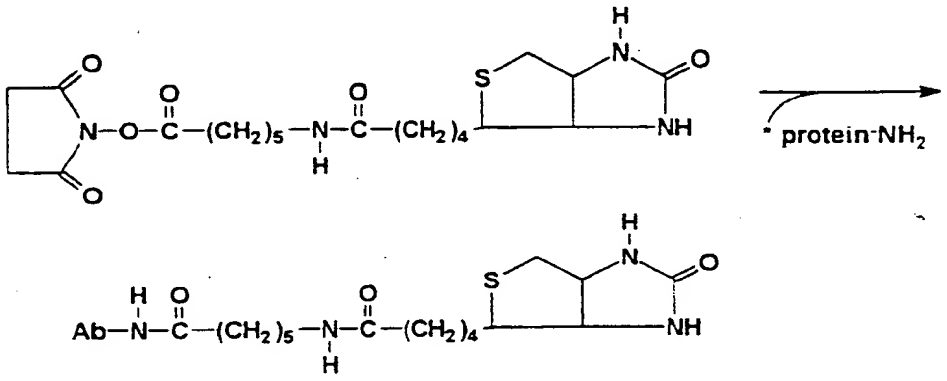
In Vivo Analysis of Radiolabeled Chelate-Biotin Conjugates Administered After Antibody Pretargeting

The ^{186}Re-chelate-biotin conjugate of Example I was studied in an animal model of a three-step antibody pretargeting protocol. Generally, this protocol involved: (i) prelocalization of biotinylated monoclonal antibody; (ii) administration of avidin for formation of a "sandwich" at the target site and for clearance of residual circulating biotinylated antibody; and (iii) administration of the 186Re-biotin conjugate for target site localization and rapid blood clearance.

A. Preparation and Characterization of Biotinylated Antibody

Biotinylated NR-LU-10 was prepared according to either of the following procedures. The first

procedure involved derivitization of antibody via lysine ε-amino groups. NR-LU-10 was radioiodinated at tyrosines using chloramine T and either ^{125}I or ^{131}I sodium iodide. The radioiodinated antibody (5-10 mg/ml) was then biotinylated using biotinamido caproate NHS ester in carbonate buffer, pH 8.5, containing 5% DMSO, according to the scheme below.



The impact of lysine biotinylation on antibody immunoreactivity was examined. As the molar offering of biotin:antibody increased from 5:1 to 40:1, biotin incorporation increased as expected (measured using the HABA assay and pronase-digested product) (Table 2, below). Percent of biotinylated antibody immunoreactivity as compared to native antibody was assessed in a limiting antigen ELISA assay. The immunoreactivity percentage dropped below 70% at a measured derivitization of 11.1:1; however, at this level of derivitization, no decrease was observed in antigen-positive cell binding (performed with LS-180 tumor cells at antigen excess). Subsequent experiments used antibody derivatized at a biotin:antibody ratio of 10:1.

TABLE 2

Effect of Lysine Biotinylation on Immunoreactivity

Molar Offering (Biotins/Ab)	Measured Derivitization (Biotins/Ab)	Immunoassessment (%) ELISA	Cell Binding
5:1	3.4	86	
10:1	8.5	73	100
13:1	11.1	69	102
20:1	13.4	36	106
40:1	23.1	27	

Alternatively, NR-LU-10 was biotinylated using thiol groups generated by reduction of cystines. Derivitization of thiol groups was hypothesized to be less compromising to antibody immunoreactivity. NR-LU-10 was radioiodinated using p-aryltin phenylate NHS ester (PIP-NHS) and either ^{125}I or ^{131}I sodium iodide. Radioiodinated NR-LU-10 was incubated with 25 mM dithiothreitol and purified using size exclusion chromatography. The reduced antibody (containing free thiol groups) was then reacted with a 10- to 100-fold molar excess of N-iodoacetyl-n'-biotinyl hexylene diamine in phosphate-buffered saline (PBS), pH 7.5, containing 5% DMSO (v/v).

TABLE 3

Effect of Thiol Biotinylation on Immunoreactivity

Molar Offering (Biotins/Ab)	Measured Derivitization (Biotins/Ab)	Immunoassessment (%) ELISA	Cell Binding
10:1	4.7	114	
50:1	6.5	102	100
100:1	6.1	95	100

As shown in Table 3, at a 50:1 or greater biotin:antibody molar offering, only 6 biotins per

antibody were incorporated. No significant impact on immunoreactivity was observed.

The lysine- and thiol-derivatized biotinylated antibodies ("antibody (lysine)" and "antibody (thiol)", respectively) were compared. Molecular sizing on size exclusion FPLC demonstrated that both biotinylation protocols yielded monomolecular (monomeric) IgGs. Biotinylated antibody (lysine) had an apparent molecular weight of 160 kD, while biotinylated antibody (thiol) had an apparent molecular weight of 180 kD. Reduction of endogenous sulfhydryls (i.e., disulfides) to thiol groups, followed by conjugation with biotin, may produce a somewhat unfolded macromolecule. If so, the antibody (thiol) may display a larger hydrodynamic radius and exhibit an apparent increase in molecular weight by chromatographic analysis. Both biotinylated antibody species exhibited 98% specific binding to immobilized avidin-agarose.

Further comparison of the biotinylated antibody species was performed using non-reducing SDS-PAGE, using a 4% stacking gel and a 5% resolving gel. Biotinylated samples were either radiolabeled or unlabeled and were combined with either radiolabeled or unlabeled avidin or streptavidin. Samples were not boiled prior to SDS-PAGE analysis. The native antibody and biotinylated antibody (lysine) showed similar migrations; the biotinylated antibody (thiol) produced two species in the 50-75 kD range. These species may represent two thiol-capped species. Under these SDS-PAGE conditions, radiolabeled streptavidin migrates as a 60 kD tetramer. When 400 μg/ml radiolabeled streptavidin was combined with 50 μg/ml biotinylated antibody (analogous to "sandwiching" conditions in vivo), both antibody species formed large molecular weight complexes. However, only the biotinylated antibody (thiol)-streptavidin complex

moved from the stacking gel into the resolving gel, indicating a decreased molecular weight as compared to the biotinylated antibody (lysine)-streptavidin complex.

B. Blood Clearance of Biotinylated Antibody Species

Radioiodinated biotinylated NR-LU-10 (lysine or thiol) was intravenously administered to non-tumored nude mice at a dose of 100 μg. At 24 h post-administration of radioiodinated biotinylated NR-LU-10, mice were intravenously injected with either saline or 400 μg of avidin. With saline administration, blood clearances for both biotinylated antibody species were biphasic and similar to the clearance of native NR-LU-10 antibody.

In the animals that received avidin intravenously at 24 h, the biotinylated antibody (lysine) was cleared (to a level of 5% of injected dose) within 15 min of avidin administration (avidin:biotin = 10:1). With the biotinylated antibody (thiol), avidin administration (10:1 or 25:1) reduced the circulating antibody level to about 35% of injected dose after two hours. Residual radiolabeled antibody activity in the circulation after avidin administration was examined in vitro using immobilized biotin. This analysis revealed that 85% of the biotinylated antibody was complexed with avidin. These data suggest that the biotinylated antibody (thiol)-avidin complexes that were formed were insufficiently crosslinked to be cleared by the RES.

Blood clearance and biodistribution studies of biotinylated antibody (lysine) 2 h post-avidin or post-saline administration were performed. Avidin administration significantly reduced the level of biotinylated antibody in the blood (see Figure 1), and increased the level of biotinylated antibody in the

liver and spleen. Kidney levels of biotinylated antibody were similar.

Example V

In Vivo Characterization of ^{186}Re-Chelate-Biotin Conjugates In a Three-Step Pretargeting Protocol

A ^{186}Re-chelate-biotin conjugate of Example I (MW ≈ 1000; specific activity = 1-2 mCi/mg) was examined in a three-step pretargeting protocol in an animal model. More specifically, 18-22 g female nude mice were implanted subcutaneously with LS-180 human colon tumor xenografts, yielding 100-200 mg tumors within 10 days of implantation.

NR-LU-10 antibody (MW ≈ 150 kD) was radiolabeled with ^{125}I/Chloramine T and biotinylated via lysine residues (as described in Example IV.A, above). Avidin (MW ≈ 66 kD) was radiolabeled with ^{131}I/PIP-NHS (as described for radioiodination of NR-LU-10 in Example IV.A., above). The experimental protocol was as follows:

Group 1:	**Time 0**, inject 100 μg ^{125}I-labeled, biotinylated NR-LU-10 **Time 24 h**, inject 400 μg ^{131}I-labeled avidin **Time 26 h**, inject 60 μg ^{186}Re-chelate-biotin conjugate
Group 2: (control)	**Time 0**, inject 400 μg ^{131}I-labeled avidin **Time 2 h**, inject 60 μg ^{186}Re-chelate-biotin conjugate
Group 3: (control)	**Time 0**, inject 60 μg ^{186}Re-chelate-biotin conjugate

The three radiolabels employed in this protocol are capable of detection in the presence of each other. It is also noteworthy that the sizes of the three elements involved are logarithmically different -- antibody ≅ 150,000; avidin ≅ 66,000; and biotin ≅ 1,000. Biodistribution analyses were performed at 2,

6, 24, 72 and 120 h after administration of the ^{186}Re-chelate-biotin conjugate.

Certain preliminary studies were performed in the animal model prior to analyzing the ^{186}Re-chelate-biotin conjugate in a three-step pretargeting protocol. First, the effect of biotinylated antibody on blood clearance of avidin was examined. These experiments showed that the rate and extent of avidin clearance was similar in the presence or absence of biotinylated antibody. Second, the effect of biotinylated antibody and avidin on blood clearance of the ^{186}Re-chelate-biotin conjugate was examined; blood clearance was similar in the presence or absence of biotinylated antibody and avidin. Further, antibody immunoreactivity was found to be uncompromised by biotinylation at the level tested.

Third, tumor uptake of biotinylated antibody administered at time 0 or of avidin administered at time 24 h was examined. The results of this experimentation are shown in Fig. 1. At 25 h, about 350 pmol/g biotinylated antibody was present at the tumor; at 32 h the level was about 300 pmol/g; at 48 h, about 200 pmol/g; and at 120 h, about 100 pmol/g. Avidin uptake at the same time points was about 250, 150, 50 and 0 pmol/g, respectively. From the same experiment, tumor to blood ratios were determined for biotinylated antibody and for avidin. From 32 h to 120 h, the ratios of tumor to blood were very similar.

Rapid and efficient removal of biotinylated antibody from the blood by complexation with avidin was observed. Within two hours of avidin administration, a 10-fold reduction in blood pool antibody concentration was noted (Fig. 1), resulting in a sharp increase in tumor to blood ratios. Avidin is cleared rapidly, with greater than 90% of the injected dose cleared from the blood within 1 hour after administration. The Re-186-biotin chelate is

also very rapidly cleared, with greater than 99% of the injected dose cleared from the blood by 1 hour after administration.

The three-step pretargeting protocol (described for Group 1, above) was then examined. More specifically, tumor uptake of the ^{186}Re-chelate-biotin conjugate in the presence or absence of biotinylated antibody and avidin was determined. In the absence of biotinylated antibody and avidin, the ^{186}Re-chelate-biotin conjugate displayed a slight peak 2 h post-injection, which was substantially cleared from the tumor by about 5 h. In contrast, at 2 h post-injection in the presence of biotinylated antibody and avidin (specific), the ^{186}Re-chelate-biotin conjugate reached a peak in tumor approximately 7 times greater than that observed in the absence of biotinylated antibody and avidin. Further, the specifically bound ^{186}Re-chelate-biotin conjugate was retained at the tumor at significant levels for more than 50 h. Tumor to blood ratios determined in the same experiment increased significantly over time (i.e., T:B = $\approx$ 8 at 30 h; $\approx$ 15 at 100 h; $\approx$ 35 at 140 h).

Tumor uptake of the ^{186}Re-chelate-biotin conjugate has further been shown to be dependent on the dose of biotinylated antibody administered. At 0 μg of biotinylated antibody, about 200 pmol/g of ^{186}Re-chelate-biotin conjugate was present at the tumor at 2 h after administration; at 50 μg antibody, about 500 pmol/g of ^{186}Re-chelate-biotin conjugate; and at 100 μg antibody, about 1,300 pmol/g of ^{186}Re-chelate-biotin conjugate.

Rhenium tumor uptake via the three-step pretargeting protocol was compared to tumor uptake of the same antibody radiolabeled through chelate covalently attached to the antibody (conventional procedure). The results of this comparison are depicted in Figure 2. Blood clearance and tumor

uptake were compared for the chelate directly labeled rhenium antibody conjugate and for the three-step pretargeted sandwich. Areas under the curves (AUC) and the ratio of AUC_{tumor}/AUC_{blood} were determined. For the chelate directly labeled rhenium antibody conjugate, the ratio of AUC_{tumor}/AUC_{blood} = 24055/10235 or 2.35; for the three-step pretargeted sandwich, the ratio of AUC_{tumor}/AUC_{blood} = 46764/6555 or 7.13.

Tumor uptake results are best taken in context with radioactivity exposure to the blood compartment, which directly correlates with bone marrow exposure. Despite the fact that 100-fold more rhenium was administered to animals in the three-step protocol, the very rapid clearance of the small molecule (Re-186-biotin) from the blood minimizes the exposure to Re-186 given in this manner. In the same matched antibody dose format, direct labeled (conventional procedure) NR-LU-10 whole antibody yielded greater exposure to rhenium than did the 100-fold higher dose given in the three-step protocol. A clear increase in the targeting ratio (tumor exposure to radioactivity:blood exposure to radioactivity--AUC_{tumor}:AUC_{blood}) was observed for three-step pretargeting (approximately 7:1) in comparison to the direct labeled antibody approach (approximately 2.4:1).

Example VI

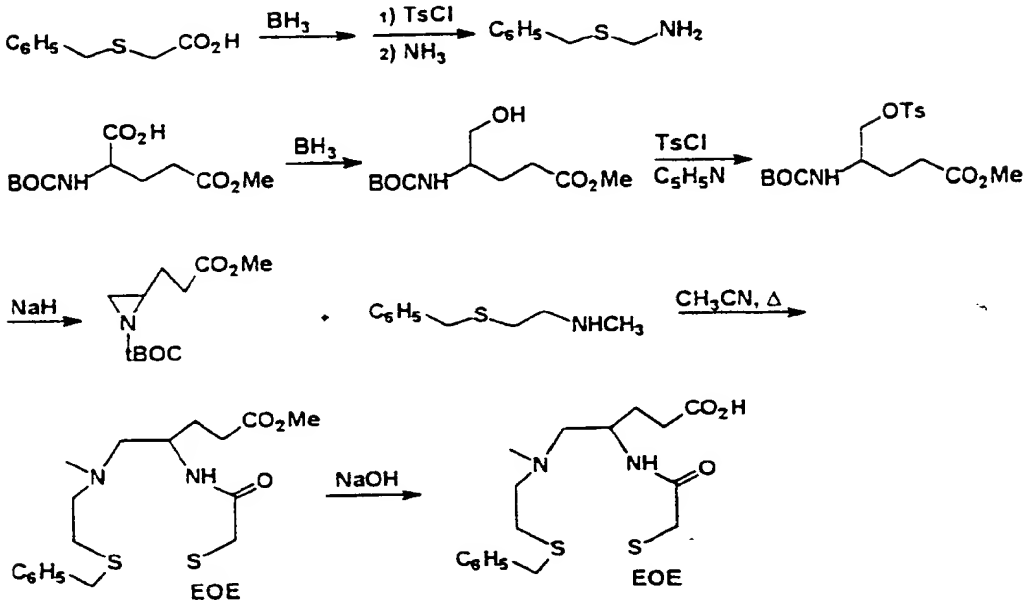
Preparation of Chelate-Biotin Conjugates Having Improved Biodistribution Properties

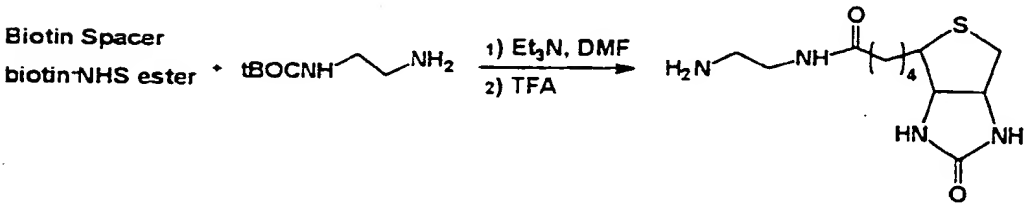
The biodistribution of ^{111}In-labeled-biotin derivatives varies greatly with structural changes in the chelate and the conjugating group. Similar structural changes may affect the biodistribution of technetium- and rhenium-biotin conjugates. Accordingly, methods for preparing technetium- and rhenium-biotin conjugates having optimal clearance from normal tissue are advantageous.

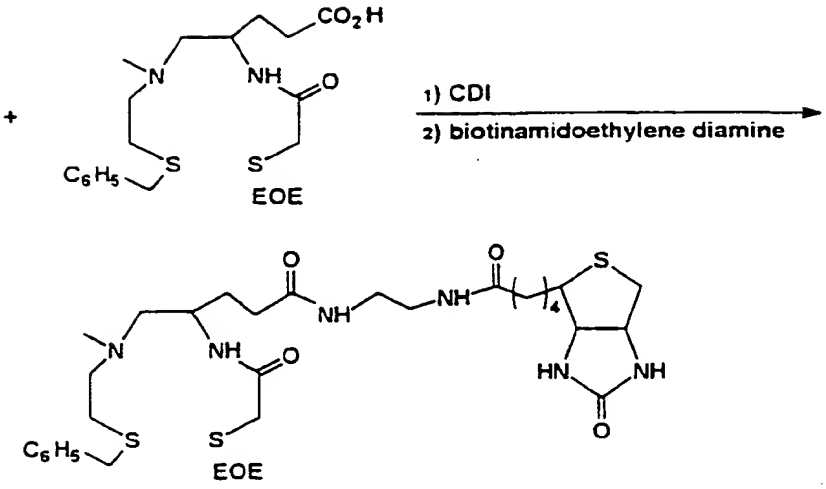
A. Neutral MAMA Chelate/Conjugate

A neutral MAMA chelate-biotin conjugate is prepared according to the following scheme.

a) MAMA ligand





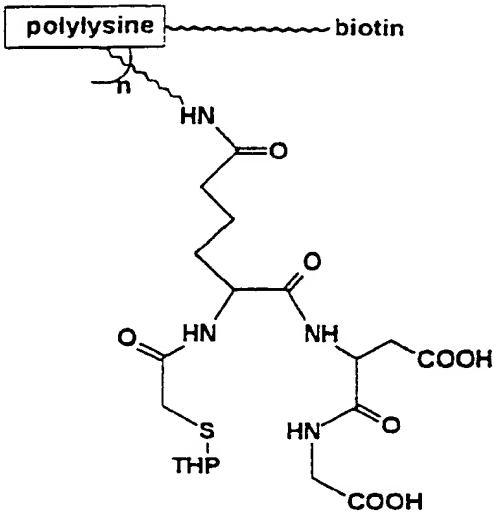


The resultant chelate-biotin conjugate shows superior kidney excretion. Although the net overall charge of the conjugate is neutral, the polycarboxylate nature of the molecule generates regions of hydrophilicity and hydrophobicity. By altering the number and nature of the carboxylate groups within the conjugate, excretion may be shifted from kidney to gastrointestinal routes. For instance, neutral compounds are generally cleared by the kidneys; anionic compounds are generally cleared through the GI system.

B. Polylysine Derivitization

Conjugates containing polylysine may also exhibit beneficial biodistribution properties. With whole antibodies, derivitization with polylysine may skew the biodistribution of conjugate toward liver uptake. In contrast, derivitization of Fab fragments with polylysine results in lower levels of both liver and

kidney uptake; blood clearance of these conjugates is similar to that of Fab covalently linked to chelate. An exemplary polylysine derivatized chelate-biotin conjugate is illustrated below.



Inclusion of polylysine in radiometal-chelate-biotin conjugates is therefore useful for minimizing or eliminating RES sequestration while maintaining good liver and kidney clearance of the conjugate. For improved renal excretion properties, polylysine derivatives are preferably succinylated following biotinylation. Polylysine derivatives offer the further advantages of: (1) increasing the specific activity of the radiometal-chelate-biotin conjugate; (2) permitting control of rate and route of blood clearance by varying the molecular weight of the polylysine polymer; and (3) increasing the circulation half-life of the conjugate for optimal tumor interaction.

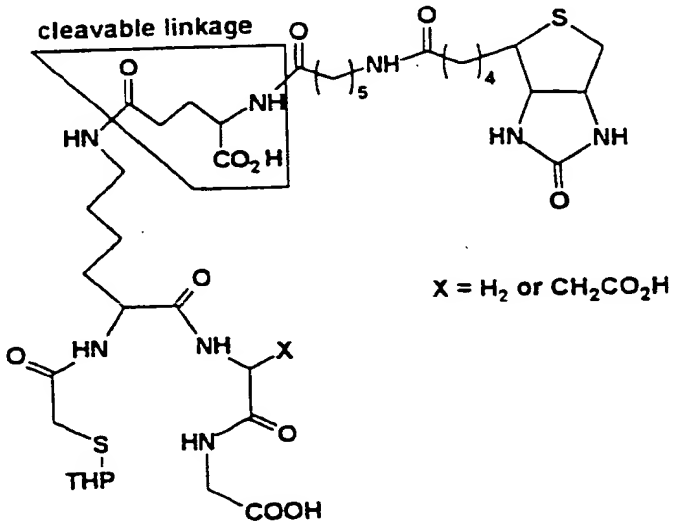
Polylysine derivitization is accomplished by standard methodologies. Briefly, poly-L-lysine is acylated according to standard amino group acylation procedures (aqueous bicarbonate buffer, pH 8, added biotin-NHS ester, followed by chelate NHS ester). Alternative methodology involves anhydrous conditions using nitrophenyl esters in DMSO and triethyl amine.

The resultant conjugates are characterized by UV and NMR spectra.

The number of biotins attached to polylysine is determined by the HABA assay. Spectrophotometric titration is used to assess the extent of amino group derivitization. The radiometal-chelate-biotin conjugate is characterized by size exclusion.

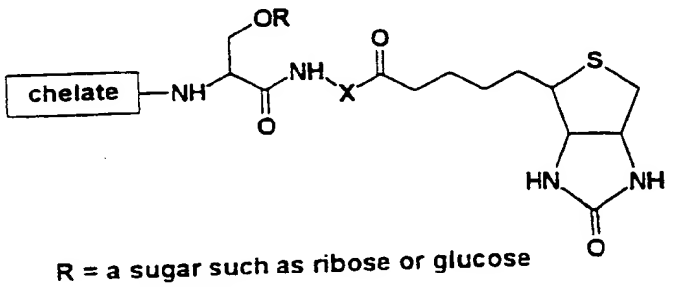
C. Cleavable Linkage

Through insertion of a cleavable linker between the chelate and biotin portion of a radiometal-chelate-biotin conjugate, retention of the conjugate at the tumor relative to normal tissue may be enhanced. More specifically, linkers that are cleaved by enzymes present in normal tissue but deficient or absent in tumor tissue can increase tumor retention. As an example, the kidney has high levels of γ-glutamyl transferase; other normal tissues exhibit in vivo cleavage of γ-glutamyl prodrugs. In contrast, tumors are generally deficient in enzyme peptidases. The glutamyl-linked biotin conjugate depicted below is cleaved in normal tissue and retained in the tumor.



D. Serine Linker With O-Polar Substituent

Sugar substitution of N_3S chelates renders such chelates water soluble. Sulfonates, which are fully ionized at physiological pH, improve water solubility of the chelate-biotin conjugate depicted below.



R = a sugar such as ribose or glucose or SO_2OH

X = $(CH_2)_0$ or $CO(CH_2)_4$

This compound is synthesized according to the standard reaction procedures. Briefly, biocytin is condensed with N-t-BOC-(O-sulfonate or O-glucose) serine NHS ester to give N-t-BOC-(O-sulfonate or O-glucose) serine biocytinamide. Subsequent cleavage of the N-t-BOC group with TFA and condensation with ligand NHS ester in DMF with triethylamine provides ligand-amidoserine(O-sulfonate or O-glucose)biocytinamide.

Example VII

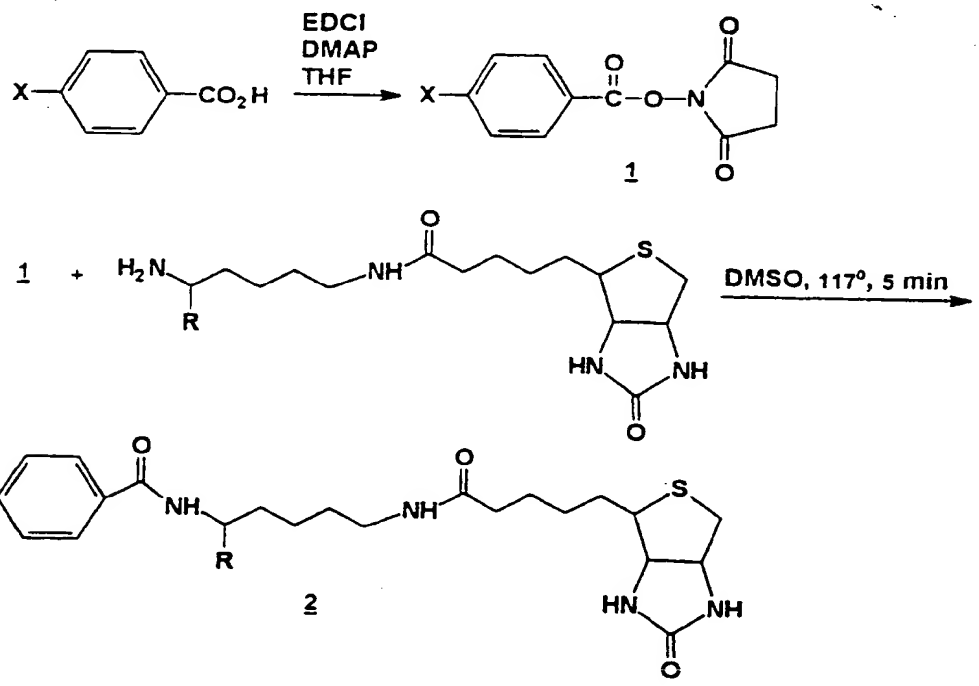
Preparation and Characterization of PIP-Radioiodinated Biotin

Radioiodinated biotin derivatives prepared by exposure of poly-L-lysine to excess NHS-LC-biotin and then to Bolton-Hunter N-hydroxysuccinimide esters in DMSO has been reported. After purification, this product was radiolabeled by the iodogen method (see, for instance, Del Rosario et al., J. Nucl. Med. 32:5, 1991, 993 (abstr.)). Because of the high molecular weight of the resultant radioiodinated biotin derivative, only limited characterization of product

(i.e., radio-HPLC and binding to immobilized streptavidin) was possible.

Preparation of radioiodinated biotin according to the present invention provides certain advantages. First, the radioiodobiotin derivative is a low molecular weight compound that is amenable to complete chemical characterization. Second, the disclosed methods for preparation involve a single step and eliminate the need for a purification step.

Briefly, iodobenzamide derivatives corresponding to biocytin (R = COOH) and biotinamidopentylamine (R = H) were prepared according to the following scheme. In this scheme, "X" may be any radiohalogen, including ^{125}I, ^{131}I, ^{123}I, ^{211}At and the like.



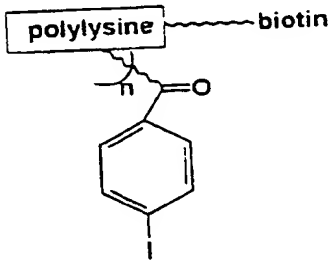
Preparation of 1 was generally according to Wilbur et al., J. Nucl. Med. 30:216-26, 1989, using a tributyltin intermediate. Water soluble carbodiimide was used in the above-depicted reaction, since the NHS ester 1 formed intractable mixtures with DCU. The NHS ester was not compatible with chromatography; it was insoluble in organic and aqueous solvents and did not

react with biocytin in DMF or in buffered aqueous acetonitrile. The reaction between 1 and biocytin or 5-(biotinamido) pentylamine was sensitive to base. When the reaction of 1 and biocytin or the pentylamine was performed in the presence of triethylamine in hot DMSO, formation of more than one biotinylated product resulted. In contrast, the reaction was extremely clean and complete when a suspension of 1 and biocytin (4 mg/ml) or the pentylamine (4 mg/ml) was heated in DMSO at 117°C for about 5 to about 10 min. The resultant ^{125}I-biotin derivatives were obtained in 94% radiochemical yield. Optionally, the radioiodinated products may be purified using C-18 HPLC and a reverse phase hydrophobic column. Hereinafter, the resultant radioiodinated products 2 are referred to as PIP-biocytin (R = COOH) and PIP-pentylamine (R = H).

Both iodobiotin derivatives 2 exhibited ≥95% binding to immobilized avidin. Incubation of the products 2 with mouse serum resulted in no loss of the ability of 2 to bind to immobilized avidin. Biodistribution studies of 2 in male BALB/c mice showed rapid clearance from the blood (similar to ^{186}Re-chelate-biotin conjugates described above). The radioiodobiotin 2 had decreased hepatobiliary excretion as compared to the ^{186}Re-chelate-biotin conjugate; urinary excretion was increased as compared to the ^{186}Re-chelate-biotin conjugate. Analysis of urinary metabolites of 2 indicated deiodination and cleavage of the biotin amide bond; the metabolites showed no binding to immobilized avidin. In contrast, metabolites of the ^{186}Re-chelate-biotin conjugate appear to be excreted in urine as intact biotin conjugates. Intestinal uptake of 2 is <50% that of the ^{186}Re-chelate-biotin conjugate. These biodistribution properties of 2 provided enhanced whole body clearance of radioisotope and indicate the advantageous use of 2 within pretargeting protocols.

^{131}I-PIP-biocytin was evaluated in a two-step pretargeting procedure in tumor-bearing mice. Briefly, female nude mice were injected subcutaneously with LS-180 tumor cells; after 7 d, the mice displayed 50-100 mg tumor xenografts. At t = 0, the mice were injected with 200 μg of NR-LU-10-streptavidin conjugate labeled with ^{125}I using PIP-NHS (see Example IV.A.). At t = 36 h, the mice received 42 μg of ^{131}I-PIP-biocytin. The data showed immediate, specific tumor localization, corresponding to ≈ 1.5 ^{131}I-PIP-biocytin molecules per avidin molecule.

The described radiohalogenated biotin compounds are amenable to the same types of modifications described in Example VI above for ^{186}Re-chelate-biotin conjugates. In particular, the following PIP-polylysine-biotin molecule is made by trace labeling polylysine with ^{125}I-PIP, followed by extensive biotinylation of the polylysine.



Assessment of ^{125}I binding to immobilized avidin ensures that all radioiodinated species also contain at least an equivalent of biotin.

Example VIII

Preparation of Biotinylated Antibody (Thiol) Through Endogenous Antibody Sulfhydryl Groups Or Sulfhydryl-G n rating Compounds

Certain antibodies have available for reaction endogenous sulfhydryl groups. If the antibody to be biotinylated contains endogenous sulfhydryl groups, such antibody is reacted with N-iodoacetyl-n'-biotinyl

hexylene diamine (as described in Example IV.A., above). The availability of one or more endogenous sulfhydryl groups obviates the need to expose the antibody to a reducing agent, such as DTT, which can have other detrimental effects on the biotinylated antibody.

Alternatively, one or more sulfhydryl groups are attached to a targeting moiety through the use of chemical compounds or linkers that contain a terminal sulfhydryl group. An exemplary compound for this purpose is iminothiolane. As with endogenous sulfhydryl groups (discussed above), the detrimental effects of reducing agents on antibody are thereby avoided.

Example IX

Two-Step Pretargeting Methodology That Does Not Induce Internalization

A NR-LU-13-avidin conjugate is prepared as follows. Initially, avidin is derivatized with N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). SMCC-derived avidin is then incubated with NR-LU-13 in a 1:1 molar ratio at pH 8.5 for 16 h. Unreacted NR-LU-13 and SMCC-derived avidin are removed from the mixture using preparative size exclusion HPLC. Two conjugates are obtained as products -- the desired 1:1 NR-LU-13-avidin conjugate as the major product; and an incompletely characterized component as the minor product.

A ^{99m}Tc-chelate-biotin conjugate is prepared as in Example II, above. The NR-LU-13-avidin conjugate is administered to a recipient and allowed to clear from the circulation. One of ordinary skill in the art of radioimmunoscintigraphy is readily able to determine the optimal time for NR-LU-13-avidin conjugate tumor localization and clearance from the circulation. At such time, the ^{99m}Tc-chelate-biotin conjugate is administered to the recipient. Because the ^{99m}Tc-

chelate-biotin conjugate has a molecular weight of ≈ 1,000, crosslinking of NR-LU-13-avidin molecules on the surface of the tumor cells is dramatically reduced or eliminated. As a result, the ^{99m}Tc diagnostic agent is retained at the tumor cell surface for an extended period of time. Accordingly, detection of the diagnostic agent by imaging techniques is optimized; further, a lower dose of radioisotope provides an image comparable to that resulting from the typical three-step pretargeting protocol.

Optionally, clearance of NR-LU-13-avidin from the circulation may be accelerated by plasmapheresis in combination with a biotin affinity column. Through use of such column, circulating NR-LU-13-avidin will be retained extracorporeally, and the recipient's immune system exposure to a large, proteinaceous immunogen (i.e., avidin) is minimized.

Exemplary methodology for plasmapheresis/column purification useful in the practice of the present invention is discussed in the context of reducing radiolabeled antibody titer in imaging and in treating tumor target sites in U.S. Patent 5,078,673. Briefly, for the purposes of the present invention, an example of an extracorporeal clearance methodology may include the following steps:

administering a ligand- or anti-ligand-targeting moiety conjugate to a recipient;

after a time sufficient for localization of the administered conjugate to the target site, withdrawing blood from the recipient by, for example, plasmapheresis;

separating cellular element from said blood to produce a serum fraction and returning the cellular elements to the recipient; and

reducing the titer of the administered conjugate in the serum fraction to produce purified serum;

infusing the purified serum back into the recipient.

Clearance of NR-LU-13-avidin is also facilitated by administration of a particulate-type clearing agent (e.g., a polymeric particle having a plurality of biotin molecules bound thereto). Such a particulate clearing agent preferably constitutes a biodegradable polymeric carrier having a plurality of biotin molecules bound thereto. Particulate clearing agents of the present invention exhibit the capability of binding to circulating administered conjugate and removing that conjugate from the recipient. Particulate clearing agents of this aspect of the present invention may be of any configuration suitable for this purpose. Preferred particulate clearing agents exhibit one or more of the following characteristics:

- microparticulate (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, with from about 0.5 to about 2 micrometers more preferred), free flowing powder structure;

- biodegradable structure designed to biodegrade over a period of time between from about 3 to about 180 days, with from about 10 to about 21 days more preferred, or non-biodegradable structure;

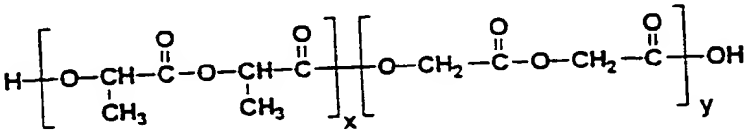
- biocompatible with the recipients physiology over the course of distribution, metabolism and excretion of the clearing agent, more preferably including biocompatible biodegradation products;

- and capability to bind with one or more circulating conjugates to facilitate the elimination or removal thereof from the recipient through one or more binding moieties (preferably, the complementary member of the ligand/anti-ligand pair). The total molar binding capacity of the particulate clearing agents depends upon the particle size selected and the ligand or anti-ligand substitution ratio. The binding

moieties are capable of coupling to the surface structure of the particulate dosage form through covalent or non-covalent modalities as set forth herein to provide accessible ligand or anti-ligand for binding to its previously administered circulating binding pair member.

Preferable particulate clearing agents of the present invention are biodegradable or non-biodegradable microparticulates. More preferably, the particulate clearing agents are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning.

Polymers derived from the condensation of alpha hydroxycarboxylic acids and related lactones are more preferred for use in the present invention. A particularly preferred moiety is formed of a mixture of thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components, such as poly(lactide-co-glycolide). An exemplary structure, a random poly(DL-lactide-co-glycolide), is shown below, with the values of x and y being manipulable by a practitioner in the art to achieve desirable microparticulate properties.



Other agents suitable for forming particulate clearing agents of the present invention include polyorthoesters and polyacetals (Polymer Letters, 18:293, 1980) and polyorthocarbonates (U.S. Patent No. 4,093,709) and the like.

Preferred lactic acid/glycolic acid polymer containing matrix particulates of the present invention are prepared by emulsion-based processes, that constitute modified solvent extraction processes such as those described by Cowsar et al., "Poly(Lactide-Co-Glycolide) Microcapsules for Controlled Release of Steroids," Methods Enzymology, 112:101-116, 1985 (steroid entrapment in microparticulates); Eldridge et al., "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies," Infection and Immunity, 59:2978-2986, 1991 (toxoid entrapment); Cohen et al., "Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres," Pharmaceutical Research, 8(6):713-720, 1991 (enzyme entrapment); and Sanders et al., "Controlled Release of a Luteinizing Hormone-Releasing Hormone Analogue from Poly(D,L-Lactide-Co-Glycolide) Microspheres," J. Pharmaceutical Science, 73(9):1294-1297, 1984 (peptide entrapment).

In general, the procedure for forming particulate clearing agents of the present invention involves dissolving the polymer in a halogenated hydrocarbon solvent and adding an additional agent that acts as a solvent for the halogenated hydrocarbon solvent but not for the polymer. The polymer precipitates out from the polymer-halogenated hydrocarbon solution. Following particulate formation, they are washed and hardened with an organic solvent. Water washing and aqueous non-ionic surfactant washing steps follow, prior to drying at room temperature under vacuum.

For biocompatibility purposes, particulate clearing agents are sterilized prior to packaging, storage or administration. Sterilization may be conducted in any convenient manner therefor. For

example, the particulates can be irradiated with gamma radiation, provided that exposure to such radiation does not adversely impact the structure or function of the binding moiety attached thereto. If the binding moiety is so adversely impacted, the particulate clearing agents can be produced under sterile conditions.

The preferred lactide/glycolide structure is biocompatible with the mammalian physiological environment. Also, these preferred sustained release dosage forms have the advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

Functional groups required for binding moiety - particulate bonding, are optionally included in the particulate structure, along with the non-degradable or biodegradable polymeric units. Functional groups that are exploitable for this purpose include those that are reactive with ligands or anti-ligands, such as carboxyl groups, amine groups, sulfhydryl groups and the like. Preferred binding enhancement moieties include the terminal carboxyl groups of the preferred (lactide-glycolide) polymer containing matrix or the like. A practitioner in the art is capable of selecting appropriate functional groups and monitoring conjugation reactions involving those functional groups.

Advantages garnered through the use of particulate clearing agents of the type described above are as follows:

- particles in the "micron" size range localize in the RES and liver, with galactose derivatization or charge modification enhancement methods for this capability available, and, preferably, are designed to remain in circulation for a time sufficient to perform the clearance function;

- the size of the particulates facilitates central vascular compartment retention thereof, substantially precluding equilibration into the peripheral or extravascular compartment;

- desired substituents for ligand or anti-ligand binding to the particulates can be introduced into the polymeric structure;

- ligand- or anti-ligand-particulate linkages having desired properties (e.g., serum biotinidase resistance thereby reducing the release of biotin metabolite from a particle-biotin clearing agent) and

- multiple ligands or anti-ligands can be bound to the particles to achieve optimal cross-linking of circulating targeting agent-ligand or -anti-ligand conjugate and efficient clearance of cross-linked species. This advantage is best achieved when care is taken to prevent particulate aggregation both in storage and upon in vivo administration.

Clearance of NR-LU-13-avidin may also be accelerated by an arterially inserted proteinaceous or polymeric multiloop device. A catheter-like device, consisting of thin loops of synthetic polymer or protein fibers derivatized with biotin, is inserted into a major artery (e.g., femoral artery) to capture NR-LU-13-avidin. Since the total blood volume passes through a major artery every 70 seconds, the in situ clearing device is effective to reduce circulating NR-LU-13-avidin within a short period of time. This device offers the advantages that NR-LU-13-avidin is not processed through the RES; removal of NR-LU-13-avidin is controllable and measurable; and fresh devices with undiminished binding capacity are insertable as necessary. This methodology is also useful with intraarterial administration embodiments of the present invention.

An alternative procedure for clearing NR-LU-13-avidin from the circulation without induction of

internalization involves administration of biotinylated, high molecular weight molecules, such as liposomes, IgM and other molecules that are size excluded from ready permeability to tumor sites. When such biotinylated, high molecular weight molecules aggregate with NR-LU-13-avidin, the aggregated complexes are readily cleared from the circulation via the RES.

Example X

Enhancement of Therapeutic Agent Internalization Through Avidin Crosslinking

The ability of multivalent avidin to crosslink two or more biotin molecules (or chelate-biotin conjugates) is advantageously used to improve delivery of therapeutic agents. More specifically, avidin crosslinking induces internalization of crosslinked complexes at the target cell surface.

Biotinylated NR-CO-04 (lysine) is prepared according to the methods described in Example IV.A., above. Doxorubicin-avidin conjugates are prepared by standard conjugation chemistry. The biotinylated NR-CO-04 is administered to a recipient and allowed to clear from the circulation. One of ordinary skill in the art of radioimmunotherapy is readily able to determine the optimal time for biotinylated NR-CO-04 tumor localization and clearance from the circulation. At such time, the doxorubicin-avidin conjugate is administered to the recipient. The avidin portion of the doxorubicin-avidin conjugate crosslinks the biotinylated NR-CO-04 on the cell surface, inducing internalization of the complex. Thus, doxorubicin is more efficiently delivered to the target cell.

In a first alternative protocol, a standard three-step pretargeting methodology is used to enhance intracellular delivery of a drug to a tumor target cell. By analogy to the description above, biotinylated NR-LU-05 is administered, followed by

avidin (for blood clearance and to form the middle layer of the sandwich at the target cell-bound biotinylated antibody). Shortly thereafter, and prior to internalization of the biotinylated NR-LU-05-avidin complex, a methotrexate-biotin conjugate is administered.

In a second alternative protocol, biotinylated NR-LU-05 is further covalently linked to methotrexate. Subsequent administration of avidin induces internalization of the complex and enhances intracellular delivery of drug to the tumor target cell.

In a third alternative protocol, NR-CO-04-avidin is administered to a recipient and allowed to clear from the circulation and localize at the target site. Thereafter, a polybiotinylated species (such as biotinylated poly-L-lysine, as in Example IV.B., above) is administered. In this protocol, the drug to be delivered may be covalently attached to either the antibody-avidin component or to the polybiotinylated species. The polybiotinylated species induces internalization of the (drug)-antibody-avidin-polybiotin-(drug) complex.

Example XI

Targeting Moiety-Anti-Ligand Conjugate for Two-Step Pretargeting In Vivo

A. Preparation of SMCC-derivatized streptavidin.

31 mg (0.48 μmol) streptavidin was dissolved in 9.0 ml PBS to prepare a final solution at 3.5 mg/ml. The pH of the solution was adjusted to 8.5 by addition of 0.9 ml of 0.5 M borate buffer, pH 8.5. A DMSO solution of SMCC (3.5 mg/ml) was prepared, and 477 μl (4.8 μmol) of this solution was added dropwise to the vortexing protein solution. After 30 minutes of stirring, the solution was purified by G-25 (PD-10, Pharmacia, Piscataway, New Jersey) column

chromatography to remove unreacted or hydrolyzed SMCC. The purified SMCC-derivatized streptavidin was isolated (28 mg, 1.67 mg/ml).

B. Preparation of DTT-reduced NR-LU-10. To 77 mg NR-LU-10 (0.42 μmol) in 15.0 ml PBS was added 1.5 ml of 0.5 M borate buffer, pH 8.5. A DTT solution, at 400 mg/ml (165 μl) was added to the protein solution. After stirring at room temperature for 30 minutes, the reduced antibody was purified by G-25 size exclusion chromatography. Purified DTT-reduced NR-LU-10 was obtained (74 mg, 2.17 mg/ml).

C. Conjugation of SMCC-streptavidin to DTT-reduced NR-LU-10. DTT-reduced NR-LU-10 (63 mg, 29 ml, 0.42 μmol) was diluted with 44.5 ml PBS. The solution of SMCC-streptavidin (28 mg, 17 ml, 0.42 μmol) was added rapidly to the stirring solution of NR-LU-10. Total protein concentration in the reaction mixture was 1.0 mg/ml. The progress of the reaction was monitored by HPLC (Zorbax® GF-250, available from MacMod). After approximately 45 minutes, the reaction was quenched by adding solid sodium tetrathionate to a final concentration of 5 mM.

D. Purification of conjugate. For small scale reactions, monosubstituted or disubstituted (with regard to streptavidin) conjugate was obtained using HPLC Zorbax (preparative) size exclusion chromatography. The desired monosubstituted or disubstituted conjugate product eluted at 14.0-14.5 min (3.0 ml/min flow rate), while unreacted NR-LU-10 eluted at 14.5-15 min and unreacted derivatized streptavidin eluted at 19-20 min.

For larger scale conjugation reactions, monosubstituted or disubstituted adduct is isolatable using DEAE ion exchange chromatography. After concentration of the crude conjugate mixture, free streptavidin was removed therefrom by eluting the column with 2.5% xylitol in sodium borate buffer, pH

8.6. The bound unreacted antibody and desired conjugate were then sequentially eluted from the column using an increasing salt gradient in 20 mM diethanolamine adjusted to pH 8.6 with sodium hydroxide.

E. Characterization of Conjugate.

1. HPLC size exclusion was conducted as described above with respect to small scale purification.

2. SDS-PAGE analysis was performed using 5% polyacrylamide gels under non-denaturing conditions. Conjugates to be evaluated were not boiled in sample buffer containing SDS to avoid dissociation of streptavidin into its 15 kD subunits. Two product bands were observed on the gel, which correspond to the mono- and di- substituted conjugates.

3. Immunoreactivity was assessed, for example, by competitive binding ELISA as compared to free antibody. Values obtained were within 10% of those for the free antibody.

4. Biotin binding capacity was assessed, for example, by titrating a known quantity of conjugate with p-[I-125]iodobenzoylbiocytin. Saturation of the biotin binding sites was observed upon addition of 4 equivalences of the labeled biocytin.

5. In vivo studies are useful to characterize the reaction product, which studies include, for example, serum clearance profiles, ability of the conjugate to target antigen-positive tumors, tumor retention of the conjugate over time and the ability of a biotinylated molecule to bind streptavidin conjugate at the tumor.

These data facilitate determination that the synthesis resulted in the formation of a 1:1 streptavidin-NR-LU-10 whole antibody conjugate that exhibits blood clearance properties similar to native NR-LU-10 whole antibody, and tumor uptake and retention properties at least equal to native NR-LU-10.

For example, Figure 3 depicts the tumor uptake profile of the NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody. LU-10-StrAv was radiolabeled on the streptavidin component only, giving a clear indication that LU-10-StrAv localizes to target cells as efficiently as NR-LU-10 whole antibody itself.

Example XII

Two-Step Pretargeting In Vivo

A ^{186}Re-chelate-biotin conjugate (Re-BT) of Example I (MW ≈ 1000; specific activity = 1-2 mCi/mg) and a biotin-iodine-131 small molecule, PIP-Biocytin (PIP-BT, MW approximately equal to 602; specific activity = 0.5-1.0 mCi/mg), as discussed in Example VII above, were examined in a three-step pretargeting protocol in an animal model, as described in Example V above. Like Re-BT, PIP-BT has the ability to bind well to avidin and is rapidly cleared from the blood, with a serum half-life of about 5 minutes. Equivalent results were observed for both molecules in the two-step pretargeting experiments described herein.

NR-LU-10 antibody (MW ≈ 150 kD) was conjugated to streptavidin (MW ≈ 66 kD) (as described in Example XI above) and radiolabeled with ^{125}I/PIP-NHS (as described for radioiodination of NR-LU-10 in Example IV.A., above). The experimental protocol was as follows:

Time 0 inject (i.v.) 200 μg NR-LU-10-StrAv conjugate;

Time 24-48 h inject (i.v.) 60-70 fold molar excess of radiolabeled biotinyl molecule ;

and perform biodistributions at 2, 6, 24, 72, 120 hours after injection of radiolabeled biotinyl molecule

NR-LU-10-streptavidin has shown very consistent patterns of blood clearance and tumor uptake in the LS-180 animal model. A representative profile is shown in Figure 4. When either PIP-BT or Re-BT is administered after allowing the LU-10-StrAv conjugate to localize to target cell sites for at least 24 hours, the tumor uptake of therapeutic radionuclide is high in both absolute amount and rapidity. For PIP-BT administered at 37 hours following LU-10-StrAv (I-125) administration, tumor uptake was above 500 pMOL/G at the 40 hour time point and peaked at about 700 pMOL/G at 45 hours post-LU-10-StrAv administration.

This almost instantaneous uptake of a small molecule therapeutic into tumor in stoichiometric amounts comparable to the antibody targeting moiety facilitates utilization of the therapeutic radionuclide at its highest specific activity. Also, the rapid clearance of radionuclide that is not bound to LU-10-StrAv conjugate permits an increased targeting ratio (tumor:blood) by eliminating the slow tumor accretion phase observed with directly labeled antibody conjugates. The pattern of radionuclide tumor retention is that of whole antibody, which is very persistent.

Experimentation using the two-step pretargeting approach and progressively lower molar doses of radiolabeled biotinyl molecule was also conducted. Uptake values of about 20% ID/G were achieved at no-carrier added (high specific activity) doses of radiolabeled biotinyl molecules. At less than saturating doses, circulating LU-10-StrAv was observed to bind significant amounts of administered radiolabeled biotinyl molecule in the blood compartment.

Example XIII

Asialoorosomucoid Clearing Agent and Two-Step Pretargeting

In order to maximize the targeting ratio (tumor:blood), clearing agents were sought that are capable of clearing the blood pool of targeting moiety-anti-ligand conjugate (e.g., LU-10-StrAv), without compromising the ligand binding capacity thereof at the target sites. One such agent, biotinylated asialoorosomucoid, which employs the avidin-biotin interaction to conjugate to circulating LU-10-StrAv, was tested.

A. Derivitization of orosomucoid. 10 mg human orosomucoid (Sigma N-9885) was dissolved in 3.5 ml of pH 5.5 0.1 M sodium acetate buffer containing 160 mM NaCl. 70 μl of a 2% (w/v) CaCl solution in deionized (D.I.) water was added and 11 μl of neuraminidase (Sigma N-7885), 4.6 U/ml, was added. The mixture was incubated at 37°C for 2 hours, and the entire sample was exchanged over a Centricon-10® ultrafiltration device (available from Amicon, Danvers, Massachusetts) with 2 volumes of PBS. The asialoorosomucoid and orosomucoid starting material were radiolabeled with I-125 using PIP technology, as described in Example IV above.

The two radiolabeled preparations were injected i.v. into female BALB/c mice (20-25 g), and blood clearance was assessed by serial retro-orbital eye bleeding of each group of three mice at 5, 10, 15 and 30 minutes, as well as at 1, 2 and 4 hours post-administration. The results of this experiment are shown in Figure 5, with asialoorosomucoid clearing more rapidly than its orosomucoid counterpart.

In addition, two animals receiving each compound were sacrificed at 5 minutes post-administration and limited biodistributions were performed. These results are shown in Figure 6. The most striking

aspects of these data are the differences in blood levels (78% for orosomucoid and 0.4% for asialoorosomucoid) and the specificity of uptake of asialoorosomucoid in the liver (86%), as opposed to other tissues.

B. Biotinylation of asialoorosomucoid clearing agent and orosomucoid control. 100 µl of 0.2 M sodium carbonate buffer, pH 9.2, was added to 2 mg (in 1.00 ml PBS) of PIP-125-labeled orosomucoid and to 2 mg PIP-125-labeled asialoorosomucoid. 60 µl of a 1.85 mg/ml solution of NHS-amino caproate biotin in DMSO was then added to each compound. The reaction mixtures were vortexed and allowed to sit at room temperature for 45 minutes. The material was purified by size exclusion column chromatography (PD-10, Pharmacia) and eluted with PBS. 1.2 ml fractions were taken, with fractions 4 and 5 containing the majority of the applied radioactivity (>95%). Streptavidin-agarose beads (Sigma S-1638) or -pellets were washed with PBS, and 20 µg of each biotinylated, radiolabeled protein was added to 400 µl of beads and 400 µl of PBS, vortexed for 20 seconds and centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed and the pellets were washed with 400 µl PBS. This wash procedure was repeated twice more, and the combined supernatants were assayed by placing them in a dosimeter versus their respective pellets. The values are shown below in Table 4.

TABLE 4

Compound	Supernatant	Pellet
orosomucoid	90%	10%
biotin-oroso	7.7%	92.%
asialoorosomucoid	92%	8.0%
biotin-asialo	10%	90%

C. Protein-Streptavidin Binding in vivo. Biotin-asialoorosomucoid was evaluated for the ability to couple with circulating LU-10-StrAv conjugate in vivo and to remove it from the blood. Female BALB/c mice (20-25 g) were injected i.v. with 200 μg LU-10-StrAv conjugate. Clearing agent (200 μl PBS - group 1; 400 μg non-biotinylated asialoorosomucoid - group 2; 400 μg biotinylated asialoorosomucoid - group 3; and 200 μg biotinylated asialoorosomucoid- group 4) was administered at 25 hours following conjugate administration. A fifth group received PIP-I-131-LU-10-StrAv conjugate which had been saturated prior to injection with biotin - group 5. The 400 μg dose constituted a 10:1 molar excess of clearing agent over the initial dose of LU-10-StrAv conjugate, while the 200 μg dose constituted a 5:1 molar excess. The saturated PIP-I-131-LU-10-StrAv conjugate was produced by addition of a 10-fold molar excess of D-biotin to 2 mg of LU-10-StrAv followed by size exclusion purification on a G-25 PD-10 column.

Three mice from each group were serially bled, as described above, at 0.17, 1, 4 and 25 hours (pre-injection of clearing agent), as well as at 27, 28, 47, 70 and 90 hours. Two additional animals from each group were sacrificed at 2 hours post-clearing agent administration and limited biodistributions were performed.

The blood clearance data are shown in Figure 7. These data indicate that circulating LU-10-StrAv radioactivity in groups 3 and 4 was rapidly and significantly reduced, in comparison to those values obtained in the control groups 1, 2 and 5. Absolute reduction in circulating antibody-streptavidin conjugate was approximately 75% when compared to controls.

Biodistribution data are shown in tabular form in Figure 8. The biodistribution data show reduced

levels of conjugate for groups 3 and 4 in all tissues except the liver, kidney and intestine, which is consistent with the processing and excretion of radiolabel associated with the conjugate after complexation with biotinylated asialoorosomucoid.

Furthermore, residual circulating conjugate was obtained from serum samples by cardiac puncture (with the assays conducted in serum + PBS) and analyzed for the ability to bind biotin (immobilized biotin on agarose beads), an indicator of functional streptavidin remaining in the serum. Group 1 animal serum showed conjugate radiolabel bound about 80% to immobilized biotin. Correcting the residual circulating radiolabel values by multiplying the remaining percent injected dose (at 2 hours after clearing agent administration) by the remaining percent able to bind immobilize biotin (the amount of remaining functional conjugate) leads to the graph shown in Figure 9. Administration of 200 μg biotinylated asialoorosomucoid resulted in a 50-fold reduction in serum biotin-binding capacity and, in preliminary studies in tumored animals, has not exhibited cross-linking and removal of prelocalized LU-10-StrAv conjugate from the tumor. Removal of circulating targeting moiety-anti-ligand without diminishing biotin-binding capacity at target cell sites, coupled with an increased radiation dose to the tumor resulting from an increase in the amount of targeting moiety-anti-ligand administered, results in both increased absolute rad dose to tumor and diminished toxicity to non-tumor cells, compared to what is currently achievable using conventional radioimmunotherapy.

A subsequent experiment was executed to evaluate lower doses of asialoorosomucoid-biotin. In the same animal model, doses of 50, 20 and 10 μg asialoorosomucoid-biotin were injected at 24 hours

following administration of the LU-10-StrAv conjugate. Data from animals serially bled are shown in Figure 10, and data from animals sacrificed two hours after clearing agent administration are shown in Figures 11A (blood clearance) and 11B (serum biotin-binding), respectively. Doses of 50 and 20 μg asialoorosomucoid-biotin effectively reduced circulating LU-10-StrAv conjugate levels by about 65% (Figure 11A) and, after correction for binding to immobilized biotin, left only 3% of the injected dose in circulation that possessed biotin-binding capacity, compared with about 25% of the injected dose in control animals (Figure 11B). Even at low doses (approaching 1:1 stoichiometry with circulating LU-10-StrAv conjugate), asialoorosomucoid-biotin was highly effective at reducing blood levels of circulating streptavidin-containing conjugate by an in vivo complexation that was dependent upon biotin-avidin interaction.

Example XIV

Streptavidin Anti-Ligand in Tumors

A set of female nude mice, implanted subcutaneously with LS-180 human colon carcinoma xenografts as described above, were randomized into groups of 4 animals/timepoint. The mice were intravenously injected with 200 μg of 1:1 mol/mol NR-LU-10 monoclonal antibody covalently coupled to streptavidin (MAB-STRPT), with the conjugate formed as described in Example XI above. The streptavidin portion of the conjugate was radiolabeled with paraiodophenyl (PIP) I-125, as described in Example IV above. Groups of mice were sacrificed at 26, 30, 48, 96 and 144 hours post-conjugate injection. Tissues were isolated, weighed and counted with respect to iodine radionuclide content using conventional procedures therefor.

A second set of female nude mice bearing LS-180 xenografts were also randomized into groups of 4 animals/timepoint. These mice were intravenously injected with 50 μg of NR-LU-10 monoclonal antibody radiolabeled with paraiodophenyl (PIP) I-131 (MAB), as described in Example IV above. Mice were sacrificed at 4, 24, 48, 128 and 168 hours post-radiolabeled monoclonal antibody injection. Tissues were isolated, weighed and counted with respect to iodine radionuclide content using conventional procedures therefor.

For each data set, a radioactivity standard of the injected dose was also counted, and data were reduced to a percent of the total injected dose per gram of tissue. Figure 12 shows the percent injected dose/gram of NR-LU-10-streptavidin-PIP-I-125 and NR-LU-10-PIP-I-131 in LS-180 tumors over time. The NR-LU-10-streptavidin conjugate exhibits higher tumor uptake and a longer retention time as compared to NR-LU-10 alone.

Example XV

Streptavidin Anti-Ligand in Liver

Female nude mice xenografted with LS-180 tumor cells, as discussed above, were randomized into groups of 4 animals/timepoint. Mice were intravenously injected with 50 μg of biotinylated NR-LU-10 monoclonal antibody that was non-covalently coupled (to form a complex) through biotin-streptavidin binding to 30 μg of streptavidin. Prior to complexation *in vivo*, the antibody portion of the complex was radiolabeled with I-125 using chloramine-T, and the streptavidin portion was labeled with paraiodophenyl (PIP) I-131, both of the labeling procedures having been described above. Mice were sacrificed at 4, 24, 48, 96 and 144 hours post-conjugate injection. Tissues were isolated, weighed

and counted with respect to the content of each iodine radionuclide using conventional procedures therefor.

A radioactivity standard of the injected doses of each complex component was also counted, and data were reduced to a percent of the total injected dose per gram of tissue. Figure 13 shows the percent injected dose per gram of streptavidin-PIP-I-131 (STREPT) and NR-LU-10-biotin-Chloramine-T-I-125 (MAB-BT) in liver over time. The complex localized at the liver as a single molecule; however, the processing of the individual components thereof differed in the liver. The I-131-streptavidin label showed prolonged residence in the liver, while the monoclonal antibody label (I-125) was rapidly lost.

In another liver study, female nude mice xenografted with xenografted with LS-180 tumor cells, as discussed above, and were intravenously injected with 200 μg of 1:1 mol/mol NR-LU-10 monoclonal antibody covalently coupled to streptavidin, prepared as described in Example XI above. The antibody portion of the conjugate was radiolabeled with paraiodophenyl (PIP-I-125). Twenty four hours later, the mice received an injection of 0.5 μg of paraiodophenyl (PIP I-131) biocytin. Mice were sacrificed at 28, 48, 120 and 168 hours post-conjugate injection. Tissues were isolated, weighed and counted with respect to the content of each iodine radionuclide using conventional procedures therefor.

A radioactivity standard of the injected doses of each complex component was also counted, and data were reduced to a percent of the total injected dose per gram of tissue (%ID/G). Figure 14 shows the percent injected dose per gram of streptavidin-monoclonal antibody-PIP-I-125 (STREP-MAB-I-125) and biocytin-PIP-I-131 (BT-I-131) in liver over time. When biocytin-PIP-I-131 was subsequently administered, the retention of streptavidin-bound biotin radiolabel (I-131) was

prolonged relative to the retention of the antibody-bound label (I-125) on the same moiety in the liver.

Example XVI

Tumor Uptake of PIP-Biocytin

PIP-Biocytin, as prepared and described in Example VII above, was tested to determine the fate thereof in vivo. The following data are based on experimentation with tumored nude mice (100 mg LS-180 tumor xenografts implanted subcutaneously 7 days prior to study) that received, at time 0, 200 μg of I-125 labeled NR-LU-10-Streptavidin conjugate (950 pmol), as discussed in Example XI above. At 24 hours, the mice received an i.v. injection of PIP-I-131-biocytin (40 μCi) and an amount of cold carrier PIP-I-127 biocytin corresponding to doses of 42 μg (69,767 pmol), 21 μg (34,884 pmol), 5.7 μg (9468 pmol), 2.85 μg (4734 pmol) or 0.5 μg (830 pmol). Tumors were excised and counted for radioactivity 4 hours after PIP-biocytin injection, and the tumor uptake data are shown in Figures 15A (%ID/G v. Dose) and 15B (pMOL/G v. Dose).

The three highest doses produced PIP-biocytin tumor localizations of about 600 pmol/g. Histology conducted on tissues receiving the two highest doses indicated that saturation of tumor-bound streptavidin was achieved. Equivalent tumor localization observed at the 5.7 μg dose (Figure 15B) is indicative of streptavidin saturation as well. In contrast, the two lowest doses produced lower absolute tumor localization of PIP-biocytin, despite equivalent localization of NR-LU-10-Streptavidin conjugate (tumors in all groups averaged about 40% ID/G for the conjugate).

The lowest dose group (0.5 μg) exhibited high efficiency tumor delivery of PIP-I-131-biocytin, which efficiency increased over time, as shown in Figure 16A. A peak uptake of 85.0 % ID/G was observed at the

120 hour time point (96 hours after administration of PIP-biocytin). Also, the absolute amount of PIP-biocytin, in terms of % ID, showed a continual increase in the tumor over all of the sampled time points (Figure 16B). The decrease in uptake on a % ID/G basis (Figure 16A) at the 168 hour time point resulted from significant growth of the tumors between the 120 and 168 hour time points.

In addition, Figure 17A shows the co-localization of NR-LU-10-Streptavidin conjugate (LU-10-StrAv) and the subsequently administered PIP-Biocytin at the same tumors over time. The localization of radioactivity at tumors by PIP-biocytin exhibited a pattern of uptake and retention that differed from that of the antibody-streptavidin conjugate (LU-10-StrAv). LU-10-StrAv exhibited a characteristic tumor uptake pattern that is equivalent to historical studies of native NR-LU-10 antibody, reaching a peak value of 40% ID/G between 24 and 48 hours after administration. In contrast, the PIP-Biocytin exhibited an initial rapid accretion in the tumor, reaching levels greater than those of LU-10-StrAv by 24 hours after PIP-Biocytin administration. Moreover, the localization of PIP-Biocytin continued to increase out to 96 hours, when the concentration of radioactivity associated with the conjugate has begun to decrease. The slightly greater amounts of circulating PIP-Biocytin compared to LU-10-StrAv at these time points (shown in Figure 17B) appeared insufficient to account for this phenomenon.

As Figure 18 clearly shows, the ratio of PIP-Biocytin to LU-10-StrAv in the tumor increased continually during the experiment, while the ratio in the blood decreased continually. This observation is consistent with a process involving continual binding of targeting moiety-containing conjugate (with PIP-Biocytin bound to it) from the blood to the tumor, with subsequent differential processing of the PIP-

Biocytin and the conjugate. Since radiolabel associated with the streptavidin conjugate component (compared to radiolabel associated with the targeting moiety) has shown increased retention in organs of metabolic processing (Examples XIV and XV above), PIP-Biocytin associated with the streptavidin appears to be selectively retained by the tumor cells. Because radiolabel is retained at target cell sites, a greater accumulation of radioactivity at those sites results.

The AUC_{tumor}/AUC_{blood} for PIP-Biocytin is over twice that of the conjugate (4.27 compared to 1.95, where AUC means "area under the curve"). Further, the absolute AUC_{tumor} for PIP-Biocytin is nearly twice that of the conjugate (9220 compared to 4629). Consequently, an increase in radiation dose to tumor was achieved.

Example XVII

Clearing Agent Evaluation Experimentation

The following experiments conducted on non-tumor-bearing mice were conducted using female BALB/c mice (20-25 g). For tumor-bearing mice experimentation, female nude mice were injected subcutaneously with LS-180 tumor cells, and, after 7 d, the mice displayed 50-100 mg tumor xenografts. The monoclonal antibody used in these experiments was NR-LU-10. When radiolabeled, the NR-LU-10-streptavidin conjugate was radiolabeled with I-125 using procedures described herein. When radiolabeled, PIP-biocytin was labeled with I-131 or I-125 using procedures described herein.

A. Utility of Asialoorosomucoid-Biotin (AO-Bt) in Reducing Circulating Radioactivity from a Subsequently Administered Radiolabeled Biotin Ligand. Mice bearing LS-180 colon tumor xenografts were injected with 200 micrograms NR-LU-10 antibody-streptavidin (MAb-StrAv) conjugate at time 0, which was allowed to prelocalize to tumor for 22 hours. At that time, 20 micrograms of

AO-Bt was administered to one group of animals. Two hours later, 90 micrograms of a radioisotope-bearing, ligand-containing small molecule (PIP-biotin-dextran prepared as discussed in part B hereof) was administered to this group of mice and also to a group which had not received AO-Bt. The results of this experiment with respect to radiolabel uptake in tumor and clearance from the blood indicated that tumor-targeting of the radiolabeled biotin-containing conjugate was retained while blood clearance was enhanced, leading to an overall improvement in amount delivered to target/amount located in serum. The AUC tumor/AUC blood with clearing agent was 6.87, while AUC tumor/AUC blood without clearing agent was 4.45. Blood clearance of the circulating MAb-StrAv conjugate was enhanced with the use of clearing agent. The clearing agent was radiolabeled in a separate group of animals and found to bind directly to tumor at very low levels (1.7 pmol/g at a dose of 488 total pmoles (0.35%ID/g), indicating that it does not significantly compromise the ability of tumor-bound MAb-StrAv to bind subsequently administered radiolabeled ligand.

B. Preparation Protocol for PIP-Biotin-Dextran. A solution of 3.0 mg biotin-dextran, lysine fixable (BDLF, available from Sigma Chemical Co., St. Louis, Missouri, 70,000 dalton molecular weight with approximately 18 biotins/molecule) in 0.3 ml PBS and 0.15 ml 1 M sodium carbonate, pH 9.25, was added to a dried residue (1.87 mCi) of N-succinimidyl p-I-125-iodobenzoate prepared in accordance with Wilbur, et al., J. Nucl. Med., 30: 216-226, 1989.

C. Dosing Optimization of AO-Bt. Tumored mice receiving StrAv-MAb as above, were injected with increasing doses of AO-Bt (0 micrograms, 20 micrograms, 50 micrograms, 100 micrograms and 200 micrograms). Tumor uptake of I-131-PIP-biocytin (5.7 micrograms, administered 2 hours after AO-Bt

administration) was examined. Increasing doses of AO-Bt had no effect on tumor localization of MAb-StrAv. Data obtained 44 hours after AO-Bt administration showed the same lack of effect. This data indicates that AO-Bt dose not cross-link and internalize MAb-StrAv on the tumor surface, as had been noted for avidin administered following biotinylated antibody.

PIP-biocytin tumor localization was inhibited at higher doses of AO-Bt. This effect is most likely due to reprocessing and distribution to tumor of biotin used to derivatize AO-Bt. Optimal tumor to blood ratios (% injected dose of radiolabeled ligand/gram weight of tumor divided by % injected dose of radioligand/gram weight of blood were achieved at the 50 microgram dose of AO-Bt. Biodistributions conducted following completion of the protocols employing a 50 microgram AO-Bt dose revealed low retention of radiolabel in all non-target tissues (1.2 pmol/g in blood; 3.5 pmol/gram in tail; 1.0 pmol/g in lung; 2.2 pmol/g in liver; 1.0 pmol/g is spleen; 7.0 pmol/g in stomach; 2.7 pmol/g in kidney; and 7.7 pmol/g in intestine). With 99.3 pmol/g in tumor, these results indicate effective decoupling of the PIP-biocytin biodistribution from that of the MAb-StrAv at all sites except tumor. This decoupling occurred at all clearing agent doses in excess of 50 micrograms as well. Decreases in tumor localization of PIP-biocytin was the significant result of administering clearing agent doses in excess of 50 micrograms. In addition, the amount of PIP-biocytin in non-target tissues 44 hours after administration was identical to localization resulting from administration of PIP-biocytin alone (except for tumor, where negligible accretion was seen when PIP-biocytin was administered alone), indicating effective decoupling.

D. Further Investigation of Optimal Clearing Agent Dose. Tumored mice injected with MAb-StrAv at time 0 as above; 50 micrograms of AO-Bt at time 22 hours; and 545 microcuries of I-131-PIP-biocytin at time 25 hours. Whole body radiation was measured and compared to that of animals that had not received clearing agent. 50 micrograms of AO-Bt was efficient in allowing the injected radioactivity to clear from the animals unimpeded by binding to circulating MAb-StrAv conjugate. Tumor uptake of I-131-PIP-biocytin was preserved at the 50 microgram clearing agent dose, with AUC tumor/AUC blood of 30:1 which is approximately 15-fold better than the AUC tumor/AUC blood achieved in conventional antibody-radioisotope therapy using this model.

E. Galactose- and Biotin-Derivatization of Human Serum Albumin (HSA). HSA was evaluated because it exhibits the advantages of being both inexpensive and non-immunogenic. HSA was derivatized with varying levels of biotin (1-about 9 biotins/molecule) via analogous chemistry to that previously described with respect to AO. More specifically, to a solution of HSA available from Sigma Chemical Co. (5-10 mg/ml in PBS) was added 10% v/v 0.5 M sodium borate buffer, pH 8.5, followed by dropwise addition of a DMSO solution of NHS-LC-biotin (Sigma Chemical Co.) to the stirred solution at the desired molar offering (relative molar equivalents of reactants). The final percent DMSO in the reaction mixture should not exceed 5%. After stirring for 1 hour at room temperature, the reaction was complete. A 90% incorporation efficiency for biotin on HSA was generally observed. As a result, if 3 molar equivalences of the NHS ester of LC-biotin was introduced, about 2.7 biotins per HSA molecule were obtained. Unreacted biotin reagent was removed from the biotin-derivatized HSA using G-25 size exclusion chromatography. Alternatively, the crude material may

be directly galactosylated. The same chemistry is applicable for biotinylating non-previously biotinylated dextran.

HSA-biotin was then derivatized with from 12 to 15 galactoses/molecule. Galactose derivatization of the biotinylated HSA was performed according to the procedure of Lee, et al., Biochemistry, 15: 3956, 1976. More specifically, a 0.1 M methanolic solution of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside was prepared and reacted with a 10% v/v 0.1 M NaOMe in methanol for 12 hours to generate the reactive galactosyl thioimidate. The galactosylation of biotinylated HSA began by initial evaporation of the anhydrous methanol from a 300 fold molar excess of reactive thioimidate. Biotinylated HSA in PBS, buffered with 10% v/v 0.5 M sodium borate, was added to the oily residue. After stirring at room temperature for 2 hours, the mixture was stored at 4°C for 12 hours. The galactosylated HSA-biotin was then purified by G-25 size exclusion chromatography or by buffer exchange to yield the desired product. The same chemistry is exploitable to galactosylating dextran. The incorporation efficiency of galactose on HSA is approximately 10%.

70 micrograms of Galactose-HSA-Biotin (G-HSA-B), with 12-15 galactose residues and 9 biotins, was administered to mice which had been administered 200 micrograms of StrAv-MAb or 200 microliters of PBS 24 hours earlier. Results indicated that G-HSA-B is effective in removing StrAv-MAb from circulation. Also, the pharmacokinetics of G-HSA-B is unperturbed and rapid in the presence or absence of circulating MAb-StrAv.

F. Non-Protein Clearing Agent. A commercially available form of dextran, molecular weight of 70,000 daltons, pre-derivatized with approximately 18 biotins/molecule and having an equivalent number of

free primary amines was studied. The primary amine moieties were derivatized with a galactosylating reagent, substantially in accordance with the procedure therefor described above in the discussion of HSA-based clearing agents, at a level of about 9 galactoses/molecule. The molar equivalence offering ratio of galactose to HSA was about 300:1, with about one-third of the galactose being converted to active form. 40 Micrograms of galactose-dextran-biotin (GAL-DEX-BT) was then injected i.v. into one group of mice which had received 200 micrograms MAb-StrAv conjugate intravenously 24 hours earlier, while 80 micrograms of GAL-DEX-BT was injected into other such mice. GAL-DEX-BT was rapid and efficient at clearing StrAv-MAb conjugate, removing over 66% of circulating conjugate in less than 4 hours after clearing agent administration. An equivalent effect was seen at both clearing agent doses, which correspond to 1.6 (40 micrograms) and 3.2 (80 micrograms) times the stoichiometric amount of circulating StrAv conjugate present.

G. Dose Ranging for G-HSA-B Clearing Agent. Dose ranging studies followed the following basic format:

200 micrograms MAb-StrAv conjugate administered;

24 hours later, clearing agent administered; and

2 hours later, 5.7 micrograms PIP-biocytin administered.

Dose ranging studies were performed with the G-HSA-B clearing agent, starting with a loading of 9 biotins per molecule and 12-15 galactose residues per molecule. Doses of 20, 40, 70 and 120 micrograms were administered 24 hours after a 200 microgram dose of MAb-StrAv conjugate. The clearing agent administrations were followed 2 hours later by administration of 5.7 micrograms of I-131-PIP-biocytin. Tumor uptake and blood retention of PIP-biocytin was examined 44 hours after administration

thereof (46 hours after clearing agent administration). The results showed that a nadir in blood retention of PIP-biocytin was achieved by all doses greater than or equal to 40 micrograms of G-HSA-B. A clear, dose-dependent decrease in tumor binding of PIP-biocytin at each increasing dose of G-HSA-B was present, however. Since no dose-dependent effect on the localization of MAb-StrAv conjugate at the tumor was observed, this data was interpreted as being indicative of relatively higher blocking of tumor-associated MAb-StrAv conjugate by the release of biotin from catabolized clearing agent. Similar results to those described earlier for the asialoorosomucoid clearing agent regarding plots of tumor/blood ratio were found with respect to G-HSA-B, in that an optimal balance between blood clearance and tumor retention occurred around the 40 microgram dose.

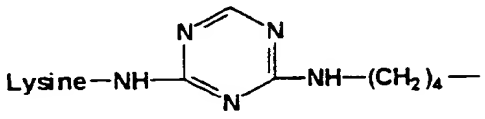
Because of the relatively large molar amounts of biotin that could be released by this clearing agent at higher doses, studies were undertaken to evaluate the effect of lower levels of biotinylation on the effectiveness of the clearing agent. G-HSA-B, derivatized with either 9, 5 or 2 biotins/molecule, was able to clear MAb-StrAv conjugate from blood at equal protein doses of clearing agent. All levels of biotinylation yielded effective, rapid clearance of MAb-StrAv from blood.

Comparison of these 9-, 5-, and 2-biotin-derivatized clearing agents with a single biotin G-HSA-B clearing agent was carried out in tumored mice, employing a 60 microgram dose of each clearing agent. This experiment showed each clearing agent to be substantially equally effective in blood clearance and tumor retention of MAb-StrAv conjugate 2 hours after clearing agent administration. The G-HSA-B with a single biotin was examined for the ability to reduce binding of a subsequently administered biotinylated

small molecule (PIP-biocytin) in blood, while preserving tumor binding of PIP-biocytin to prelocalized MAb-StrAv conjugate. Measured at 44 hours following PIP-biocytin administration, tumor localization of both the MAb-StrAv conjugate and PIP-biocytin was well preserved over a broad dose range of G-HSA-B with one biotin/molecule (90 to 180 micrograms). A progressive decrease in blood retention of PIP-biocytin was achieved by increasing doses of the single biotin G-HSA-B clearing agent, while tumor localization remained essentially constant, indicating that this clearing agent, with a lower level of biotinylation, is preferred. This preference arises because the single biotin G-HSA-B clearing agent is both effective at clearing MAb-StrAv over a broader range of doses (potentially eliminating the need for patient-to-patient titration of optimal dose) and appears to release less competing biotin into the systemic circulation than the same agent having a higher biotin loading level.

Another way in which to decrease the effect of clearing agent-released biotin on active agent-biotin conjugate binding to prelocalized targeting moiety-streptavidin conjugate is to attach the protein or polymer or other primary clearing agent component to biotin using a retention linker. A retention linker has a chemical structure that is resistant to agents that cleave peptide bonds and, optionally, becomes protonated when localized to a catabolizing space, such as a lysosome. Preferred retention linkers of the present invention are short strings of D-amino acids or small molecules having both of the characteristics set forth above. An exemplary retention linker of the present invention is cyanuric chloride, which may be interposed between an epsilon amino group of a lysine of a proteinaceous primary clearing agent component and an amine moiety of a

reduced and chemically altered biotin carboxy moiety (which has been discussed above) to form a compound of the structure set forth below.



When the compound shown above is catabolized in a catabolizing space, the heterocyclic ring becomes protonated. The ring protonation prevents the catabolite from exiting the lysosome. In this manner, biotin catabolites containing the heterocyclic ring are restricted to the site(s) of catabolism and, therefore, do not compete with active-agent-biotin conjugate for prelocalized targeting moiety-streptavidin target sites.

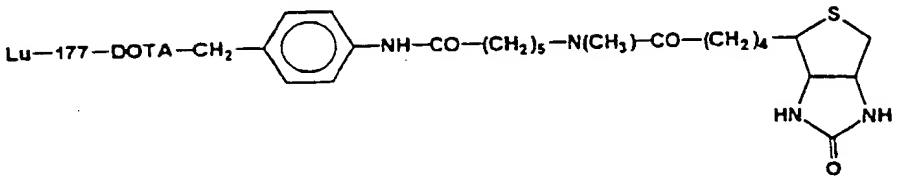
Comparisons of tumor/blood localization of radiolabeled PIP-biocytin observed in the G-HSA-B dose ranging studies showed that optimal tumor to background targeting was achieved over a broad dose range (90 to 180 micrograms), with the results providing the expectation that even larger clearing agent doses would also be effective. Another key result of the dose ranging experimentation is that G-HSA-B with an average of only 1 biotin per molecule is presumably only clearing the MAb-StrAv conjugate via the Ashwell receptor mechanism only, because too few biotins are present to cause cross-linking and aggregation of MAb-StrAv conjugates and clearing agents with such aggregates being cleared by the reticuloendothelial system.

H. Tumor Targeting Evaluation Using G-HSA-B. The protocol for this experiment was as follows:

Time 0: administer 400 micrograms MAb-StrAv conjugate;

Time 24 hours: administer 240 micrograms of G-HSA-B with one biotin and 12-15 galactoses and

Time 26 hours: administer 6 micrograms of



Lu-177 is complexed with the DOTA chelate using known techniques therefor, and the DOTA chelate is prepared in accordance with the following procedure. N-methyl-glycine (trivial name sarcosine, available from Sigma Chemical Co.) was condensed with biotin-NHS ester in DMF and triethylamine to obtain N-methyl glycyl-biotin. N-methyl-glycyl biotin was then activated with EDCI and NHS. The resultant NHS ester was not isolated and was condensed in situ with DOTA-aniline preparable using known techniques (e.g., McMurry et al., Bioconjugate Chem., 3: 108-117, 1992) and excess pyridine. The reaction solution was heated at 60°C for 10 minutes and then evaporated. The residue was purified by preparative HPLC to give [(N-methyl-N-biotinyl)-N-glycyl]-aminobenzyl-DOTA.

1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85°C during which time the solids dissolved producing a clear and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

H-NMR (DMSO): 1.18-1.25 (m, 6H, $(CH_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05-3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32

(2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23 'C for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63°C for 10 minutes, then stirred at 23°C for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30-1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70-4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).

Efficient delivery of the Lu-177-DOTA-biotin small molecule was observed, 20-25 % injected dose/gram of tumor. These values are equivalent with the efficiency of the delivery of the MAb-StrAv conjugate. The AUC tumor/AUC blood obtained for this non-optimized clearing agent dose was 300% greater than that achievable by comparable direct MAb-radiolabel administration. Subsequent experimentation has resulted in AUC tumor/AUC blood over 1000% greater than that achievable by comparable conventional MAb-radiolabel administration. In addition, the HSA-based clearing agent is expected to exhibit a low degree of immunogenicity in humans.

Example XVIII

Clearing Agent Immunosuppression

Three test groups (Groups I, II and III) of female Balb/C mice between 16 and 20 grams were used in the study, with each group containing 15 mice and split

into 3 equal subgroups containing 5 mice each. Each of the mice in the three test groups received murine NR-LU-10-streptavidin conjugate, which conjugate approximates humanized or human NR-LU-10-streptavidin conjugate administration in a human.

Group I received 120 μg NR-LU-10-streptavidin conjugate prepared substantially as described in Example XI above by intravenous injection via the tail vein in 4 weekly injections (at weeks 0, 1, 2 and 3). Group II also received 120 μg NR-LU-10-streptavidin conjugate by intravenous injection via the tail vein in 4 weekly injections. Group II mice also received 60 μg of a biotinylated and galactosylated murine serum albumin administered intravenously 24 hours after each of the 4 NR-LU-10-streptavidin conjugate administrations. Group III mice received 120 μg of NR-LU-10-streptavidin conjugate intramuscularly. Serum samples were collected on weeks 2, 4 and 6 from Groups I-III and control mice. Anti-streptavidin levels were detected by ELISA using streptavidin-coated plates and serial dilutions of serum followed by goat anti-mouse horseradish peroxidase (HRPO) detection.

The mice that received the clearing agent exhibited the lowest immune response to streptavidin compared to the mice receiving an i.m. injection of streptavidin (highest responders) or compared to the mice receiving an i.v. injection without clearing agent. Although there was variability within the experimental groups, the overall effect was a suppressed immune response.

These results suggest that the clearing agent is able to remove non-tumor associated antibody-streptavidin conjugate from circulation to the hepatobiliary system prior to the formation of anti-streptavidin antibodies by the mouse immune system. In humans, the immunosuppressive effect of the

clearing agent should be enhanced, because the levels of circulating biotin are 2 to 3 orders of magnitude lower in humans than in mice. As a result of this lower level of endogenous biotin, more circulating streptavidin will be able to bind to biotin-containing clearing agent and be rapidly cleared from circulation.

Example XIX

Administration of Monoclonal Antibody-Streptavidin in Humans

A pilot study was conducted to evaluate the biodistribution, pharmacokinetics and other parameters related to administration murine NR-LU-10-streptavidin conjugate in humans. This protocol employed a single intravenous infusion of between 25 and 125 mg/m^2 of NR-LU-10-streptavidin conjugate labeled with 25 mCi/m^2 of Re-186 in accordance with the procedure described in Breitz et al., "Clinical Experience with Rhenium-186 Labeled Monoclonal Antibodies for Radioimmunotherapy: Results of Phase I Trials," JNM, 33: 1099-1109, 1992. The infusions were well tolerated with no signs or symptoms of acute allergic reaction. Reported toxicities were clinically asymptomatic and similar to those observed in Re-186-NR-LU-10 studies.

Serum samples were taken at 10 minutes and then at 1, 2, 3, 4, 6, 8, 12, 24, 48, 72 and 120 hours following conjugate administration. To date, results in five patients show that the blood clearance of Re-186-NR-LU-10-streptavidin conjugate is similar to that of 186-Re-NR-LU-10 (historical data, n = 15). Gamma camera images were taken immediately after infusion and again at 24, 48, 72 and 120 hours following conjugate administration. Those images indicate that the Re-186-NR-LU-10-streptavidin conjugate localized to tumor in a manner similar to radiolabeled NR-LU-10 Fab (historical data).

Three patients had a tumor biopsy performed following Re-186-NR-LU-10-streptavidin infusion. Two patients had a tumor biopsy at approximately 48 hours after infusion, and one patient had a biopsy at 24 hours after infusion. Immunohistochemical analysis showed tumor localization of Re-186, antibody NR-LU-10 and streptavidin. A peroxidase-conjugated anti-murine probe was used to detect NR-LU-10 in the tumor section and a chromogen/substrate was then administered, which colorant yielded a brown stain at sites of NR-LU-10 localization. A negative result appeared as sections devoid of brown staining. A known positive tumor control (ovarian carcinoma) was also run concurrently with the clinical specimens. Degree of reactivity of clinical specimens was compared to the positive control.

Streptavidin was shown to localize in the same pattern as NR-LU-10 by rabbit-anti-streptavidin antibody which was detected by a goat anti-rabbit horse radish peroxidase conjugate (HRPO). To verify the presence of active biotin binding sites, biotin HRPO samples were developed with a chromagen substrate yielding a brown stain at sites of reactivity. Again, positive steptavidin was indicated by brown staining. These studies revealed that localized streptavidin retained biotin-binding capability.

Example XX

Immunosuppression of Monoclonal Antibody-Containing Conjugates

Fifteen patients (ages 39-78) with metastatic adenocarcinoma received cyclosporin and both an imaging and a subsequent therapeutic dose of a murine monoclonal antibody. Two of these patients also received second and third therapeutic antibody doses, and one patient received a second dose only.

NR-CO-02 is a murine IgG1 monoclonal antibody that recognizes a CEA-like antigen. NR-CO-02 was pepsin cleaved to the Fab')$_2$ fragment which was labeled with Tc-99m for imaging and Re-196 for therapy. NR-LU-10 is a murine IgG2b monoclonal antibody that recognizes a glycoprotein antigen expressed by several epithelial tumors. The Fab fragment of NR-LU-10 was labeled with Tc-99m and used for diagnostic imaging. The intact murine antibody was labeled with Re-186 and administered for radioimmunotherapy. Antibody production, isotopic labeling and quality control issues were addressed as set forth in Breitz et al., "Clinical Experience with Rhenium-186-Labeled Monoclonal Antibodies for Radioimmunotherapy: Results of Phase I Trials," J. Nucl. Med., 33: 1099-1109, 1992.

Cyclosporin was given orally at an initial dose of 8.6-15 mg/kg/day. The daily dose was divided into two doses taken 12 hours apart and begun 48 hours prior to administration of the Tc-99m-labeled murine antibody fragment. Cyclosporin was continued for 14 days after the second (Re-186-labeled) murine antibody administration. Cyclosporin serum levels were determined just prior to a morning dose (CYCLO-Trac® SP-Whole Blood Radioimmunoassay for Cyclosporine, INCSTAR Corp., Stillwater, MN). Seven patients began cyclosporin at 10 mg/kg/day had a median serum level of 457 (range: 215-638) ng/ml, while six patients who began cyclosporin at 15 mg/kg/day had a median serum levle of 742 (range: 426-1262) ng/ml.

Patients were clinically monitored for toxicity with special attention to systemic symptoms, blood pressure and renal and hepatic function, and dose reductions were undertaken as clinically indicated. Cyclosporin toxicity was common but generally mild and always readily reversible.

Serum was obtained at intervals to evaluation HAMA formation as described in Schroff et al., "Human Anti-Murine Immunoglobulin Responses in Patients Receiving Monoclonal Antibody Therapy," Cancer Res, 45: 879-885, 1985. The criterion for a positive response was the development of a HAMA titer twice a patient's baseline titer and two standard deviations above the geometric mean of a normal population (4.6 WHO units for NR-LU-10 or 10.7 WHO units for NR-CO-02). HAMA results in patients receiving cyclosporin were compared with those previously reported in 28 patients who received two doses of NR-CO-02 $F(ab')_2$ or 15 patients who received a single dose of NR-LU-10 Fab followed by a single dose of intact NR-LU-10 antibody (Breitz et al. referenced above).

Three patients received cyclosporin, 15 mg/kg/day (2 patients) or 10 mg/kg/day (1 patient), and two doses of NR-CO-02 $F(ab')_2$ antibody fragment. All patients received approximately 18 mg of $Fab')_2$ NR-CO-02 followed 8-9 days later by approximately 46 mg of Re-186 labeled $F(ab')_2$ NR-CO-02. None of the three patients tested developed a significant titer of HAMA in contrast to the formation of significant titers of HAMA by 86% of patients who received two doses of NR-CO-02 $F(ab')_2$ without cyclosporin as reported in Breitz et al. Mean serum cyclosporin levels in these three patients were relatively high (median = 726 ng/ml).

Ten patients received cyclosporin, 8.6-15 mg/kg/day, and then approximately 8 mg of Tc-99m-labeled NR-LU-10 Fab fragment. Two to Eight (median six) days later these patients were given about 40 mg of Re-186-labeled intact NR-LU-10 antibody. At weeks 2 and 4, 33-50% of 10 patients developed significant HAMA titers compared to 80-92% of 15 patients who had comparable exposure to NR-LU-10 Fab and intact NR-LU-10 without cyclosporin. By weeks 5 to 8, 80% of patients had formed significant titers of HAMA

compared to 100% of patients that received a similar regimen without cyclosporin administration. The geometric mean titer of HAMA formed by patients who were given cyclosporin was substantially less than that of patients given NR-LU-10 without cyclosporin. In general, the higher the mean cyclosporin serum level, the lower the peak HAMA titer.

Cyclosporin given from two days before until two weeks after administration can slow down or suppress HAMA formation.

Example XXI

Two-Step Pretargeting With Immunosuppression

A patient presents with colon cancer. An oral dose of cyclosporin ranging between 10 and 15 mg/kg/day is administered, preferably in two doses given 12 hours apart, for two days. A monoclonal antibody (MAb) directed to a colon cancer cell antigen such as NR-LU-10 is conjugated to streptavidin to form a NR-LU-10-streptavidin conjugate. At day three, the NR-LU-10-streptavidin conjugate is administered to the patient in an amount in excess of the maximum tolerated dose of conjugate administrable in a targeted, chelate labeled molecule protocol (e.g., administration of monoclonal antibody-chelate-radionuclide conjugate) and is permitted to localize to target cancer cells for 24-48 hours. Galactose-human serum albumin-biotin as described in Example XVIII above is then administered as a clearing agent to remove circulating NR-LU-10-streptavidin conjugate. At 2-6 hours post-clearing agent administration, Y-90-DOTA-N-methyl-glycine-biotin conjugate of the type discussed in Example XXII below is dispersed in a pharmaceutically acceptable diluent and administered to the patient in a therapeutically effective dose. The biotin-radionuclide chelate conjugate localizes to the targeted NR-LU-10-streptavidin moiety or is

removed from the patient via the renal pathway. During the pretargeting protocol and for a period of 2 weeks following NR-LU-10-streptavidin administration (days 3-18), 10-15 mg/kg/day of cyclosporin is administered, preferably in two daily administrations as described above, to reduce HAMA response to NR-LU-10 if murine or chimeric antibody is employed and/or to reduce HASA to streptavidin.

Example XXII

Non-specific Antibody Pretargeting Methods

BALB/c mice bearing SHT-1 tumors were employed in this study. The average initial tumor volume for the mice employed in this study was in excess of 250 mm^3, constituting a large tumor burden. Three groups of test mice were studied as follows:

Group I: 400 μCi of Y-90-DOTA-N-methyl-glycine-biotin, preparable in accordance with the synthetic procedure described below, was administered.

1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85°C during which time the solids dissolved producing a clear and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

H-NMR (DMSO): 1.18-1.25 (m, 6H, $(CH_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05-3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32

(2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23 'C for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63°C for 10 minutes, then stirred at 23°C for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30-1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70-4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).

Group II: At t = 0, 400 μg NR-ML-05-streptavidin (NR-ML-05 is a monoclonal antibody that is non-specific for SHT-1 tumors) was administered;

At t = 24 hours, 225 μg of galactose-human serum albumin-biotin clearing agent was administered; and

At t = 26 hours, 400 μCi of Y-90-DOTA-N-methyl-glycine-biotin was administered.

Group III: At t = 0, 400 μg NR-LU-10-streptavidin (NR-LU-10 is a monoclonal antibody that is specific for SHT-1 tumors) was administered;

At t = 24 hours, 225 μg of galactose-human serum albumin-biotin clearing agent was administered; and

At t = 26 hours, 400 μCi of Y-90-DOTA-N-methyl-glycine-biotin was administered.

Even for this large tumor burden, the mice that received NR-ML-05-streptavidin exhibited slower tumor

growth than the tumors of the Group I mice that received radiolabeled biotin only. Group II mice also exhibited tumor regressions to approximately 60% of original tumor size. The tumors began to grow slowly following the aforementioned regression. Complete or near complete tumor regressions were observed in mice given the NR-LU-10-streptavidin conjugate protocol.

Kits containing one or more of the components described above are also contemplated. For instance, radiohalogenated biotin may be provided in a sterile container for use in pretargeting procedures. A chelate-biotin conjugate provided in a sterile container is suitable for radiometallation by the consumer; such kits would be particularly amenable for use in pretargeting protocols. Alternatively, radiohalogenated biotin and a chelate-biotin conjugate may be vialed in a non-sterile condition for use as a research reagent.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WHAT IS CLAIMED IS:

1. A method of increasing active agent localization at a target cell site of a mammalian recipient, which method comprises:

administering to the recipient a first conjugate comprising a targeting moiety and streptavidin;

administering to the recipient an immunosuppressive agent prior to, concurrently with, or subsequent to the administration of the first conjugate, wherein the immunosuppressive agent renders the first conjugate less immunogenic;

allowing an amount of time to pass that is sufficient for localization of the first conjugate to a target cell site;

administering to the recipient a second conjugate comprising an active agent, biotin and a linker that is resistant to biotinidase cleavage, wherein the second conjugate localizes to target cell-bound first conjugate.

2. A method of claim 1 wherein the immunosuppressive agent is administered both prior and subsequent to first conjugate administration.

3. A method of claim 1 wherein the immunosuppressive agent is selected from the group consisting of cyclosporin A, verapamil, azathioprine, cyclophosphamide, deoxyspergualin, FK506, rapamycin and mycophenolic acid; and wherein the immunosuppressive agent is in free, liposome encapsulated, or microparticulate form.

4. A method of claim 3 wherein the immunosuppressive agent is cyclosporin A.

5. A method of claim 4 wherein cyclosporin A is administered in combination with an agent selected from the group consisting of verapamil, diltiazem, nicardipine, erythromycin, isotretinoin, metoclopramide, fluconazole, acetazolamide, methotrexate, mycophenolate and ketoconazole.

6. A method of claim 1 wherein the targeting moiety is a humanized antibody or a target site-localizing fragment thereof.

7. A method of claim 1 wherein streptavidin is recombinant streptavidin exhibiting an immunogenicity-reducing structural modification selected from the group consisting of polyethylene glycol derivatization, charge modification and tertiary structure modification.

8. A method of claim 1 wherein the active agent is selected from the group consisting of a radionuclide, a chemotherapeutic drug, an anti-tumor agent and a toxin.

9. A method of claim 8 wherein the active agent is a radionuclide selected from the group consisting of Re-186, Re-188, Y-90, At-211, Pb-212, Bi-212, Sm-153, Eu-169, Lu-177, Cu-67, Rh-105, In-111, Au-198, I-123, Tc-99m and I-131.

10. A method of claim 1 wherein the active agent is a cytokine or a lectin inflammatory response promoter.

11. A method of claim 1 wherein the step of administering the second conjugate is conducted by intralesional or intraarterial injection.

12. A method of claim 11 wherein the second conjugate is administered via an artery supplying target tissue.

13. A method of claim 11 wherein the second conjugate is administered via an artery selected from the group consisting of hepatic artery, carotid artery, bronchial artery and renal artery.

14. A method of claim 1 wherein the second conjugate is administered intravenously.

15. A method of claim 1, further comprising, between administration of the first conjugate and the second conjugate, administering to the recipient a clearing agent capable of increasing blood clearance or decreasing in vivo non-target binding capability of the first conjugate, wherein the clearing agent decreases the immunogenicity of the first conjugate.

16. A method of claim 15 wherein the clearing agent is asialoorosomucoid.

17. A method of claim 15 wherein the clearing agent has the following structure:

$(Hexose)_m$--Human Serum Albumin (HSA)--$(Ligand)_n$, wherein n is an integer from 1 to 10 and m is an integer from 1 to 25, and wherein the hexose is recognized by Ashwell receptors.

18. A method of increasing active agent localization at a target cell site of a mammalian recipient, which method comprises:

administering to the recipient a first conjugate comprising a non-specific targeting moiety and streptavidin;

allowing an amount of time to pass that is sufficient for localization of the first conjugate to a target cell site;

administering to the recipient a second conjugate comprising an active agent, biotin and a linker that is resistant to biotinidase cleavage, wherein the second conjugate localizes to target cell-bound first conjugate.

19. A method of claim 18, further comprising, between administration of the first conjugate and the second conjugate, administering to the recipient a clearing agent capable of increasing blood clearance or decreasing in vivo non-target binding capability of the first conjugate, wherein the clearing agent decreases the immunogenicity of the first conjugate.

20. A method of claim 18, further comprising administering to the recipient an immunosuppressive agent prior to, concurrently with, or subsequent to the administration of the first conjugate, wherein the immunosuppressive agent renders the first conjugate less immunogenic;

21. An article of manufacture comprising a package having a label and containing a first conjugate, wherein the first conjugate comprises a targeting moiety component that binds to an antigen recognized by a NR-LU-10 antibody and a streptavidin component;

and wherein the first conjugate is capable of localizing at a target site upon administration to a mammalian recipient, and the streptavidin component retains the ability to bind to biotin;

and further wherein the label identifies the targeting moiety component and the streptavidin component.

22. An article of manufacture of claim 21 wherein the label further identifies that the first conjugate is limited to investigational use.

23. An article of manufacture of claim 21 wherein the targeting moiety is a humanized antibody or a target site-localizing fragment thereof.

24. An article of manufacture of claim 21 wherein the first conjugate is contained within a vial.

25. An article of manufacture of claim 24 wherein the first conjugate is vialed in a sterile, pyrogen-free environment.

26. An article of manufacture of claim 22 wherein the article of manufacture is employed to diagnose or treat small cell lung cancer.

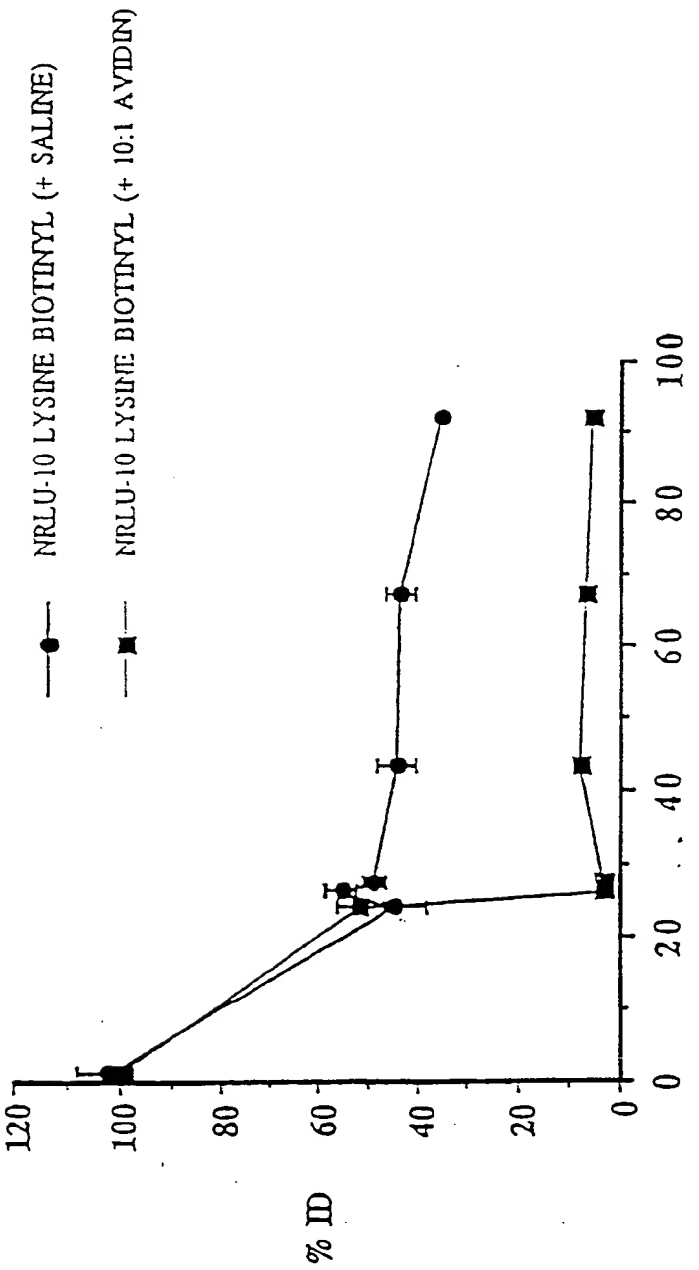

NRLU-10 LYSINE BIOTINYL (+ SALINE)
NRLU-10 LYSINE BIOTINYL (+ 10:1 AVIDIN)
% ID
120
100
80
60
40
20
0
0
20
40
60
80
100
TIME (HR)

Fig. 1.

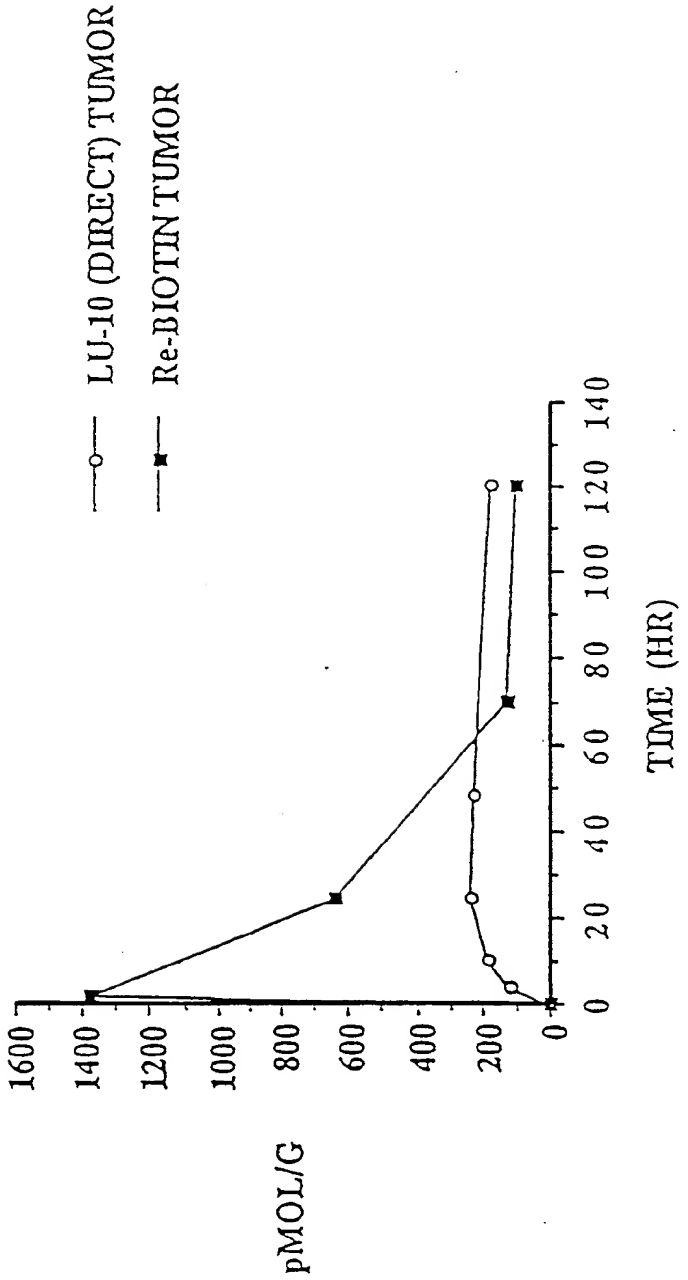

LU-10 (DIRECT) TUMOR
Re-BIOTIN TUMOR
pMOL/G
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
TIME (HR)

Fig. 2.

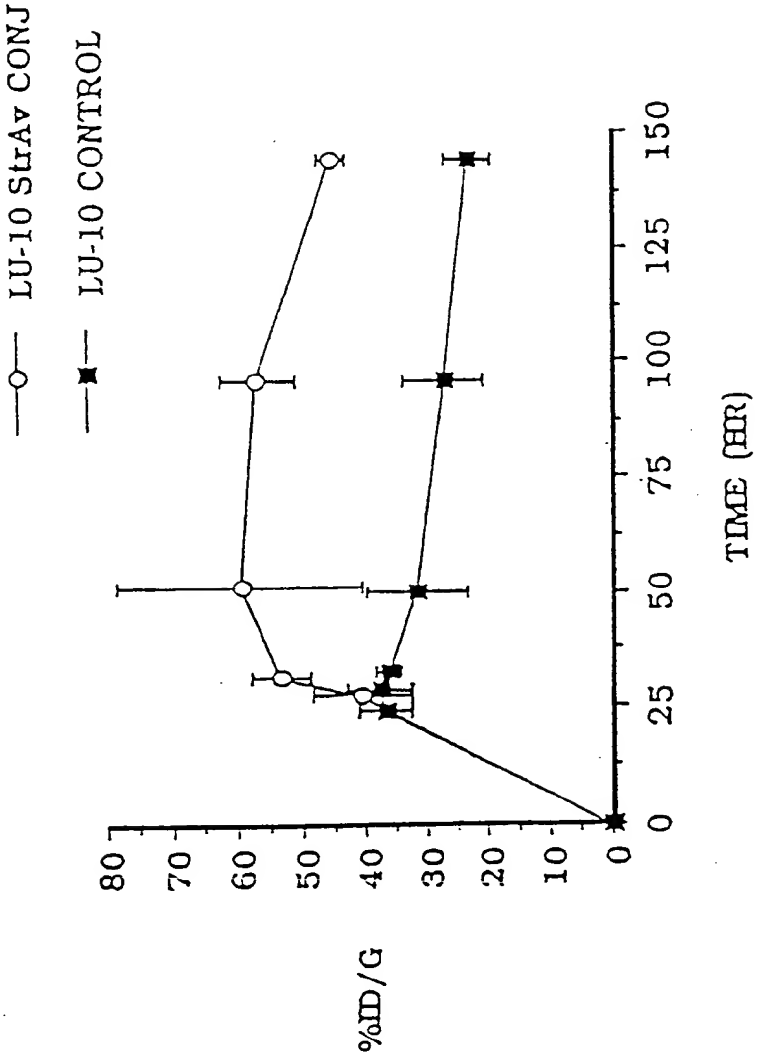

LU-10 StrAv CONJ
LU-10 CONTROL
%ID/G
80
70
60
50
40
30
20
10
0
0
25
50
75
100
125
150
TIME (HR)

Fig. 3.

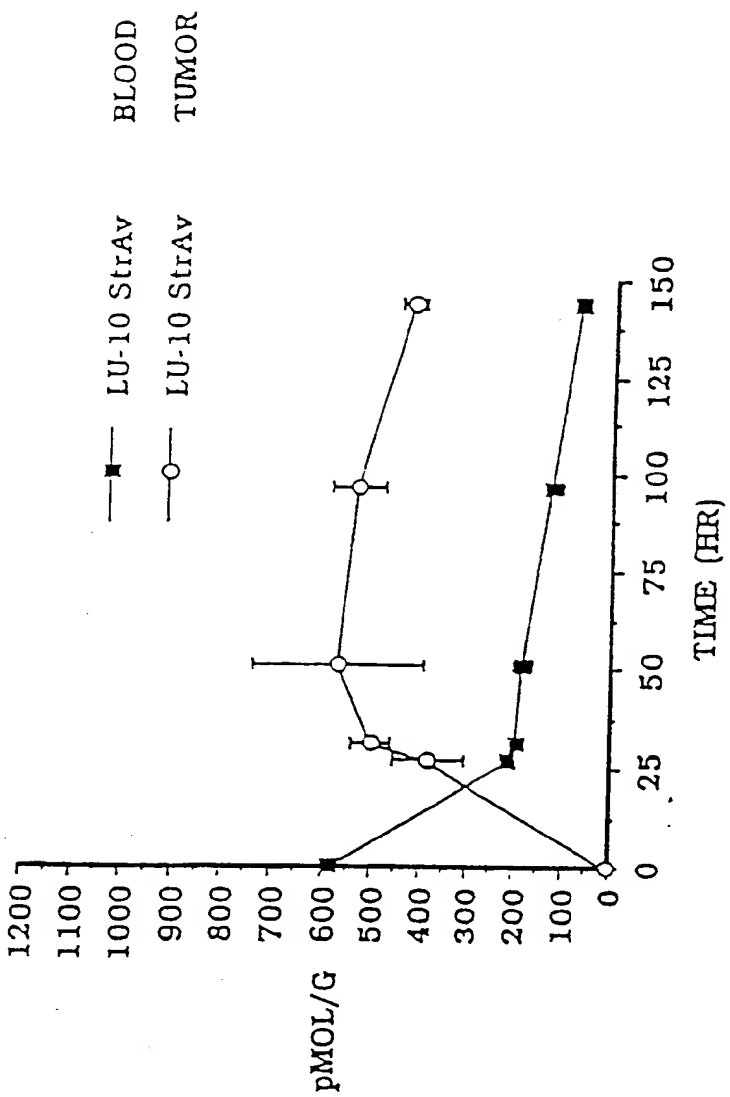

LU-10 StrAv BLOOD
LU-10 StrAv TUMOR
pMOL/G
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
25
50
75
100
125
150
TIME (HR)

Fig. 4.

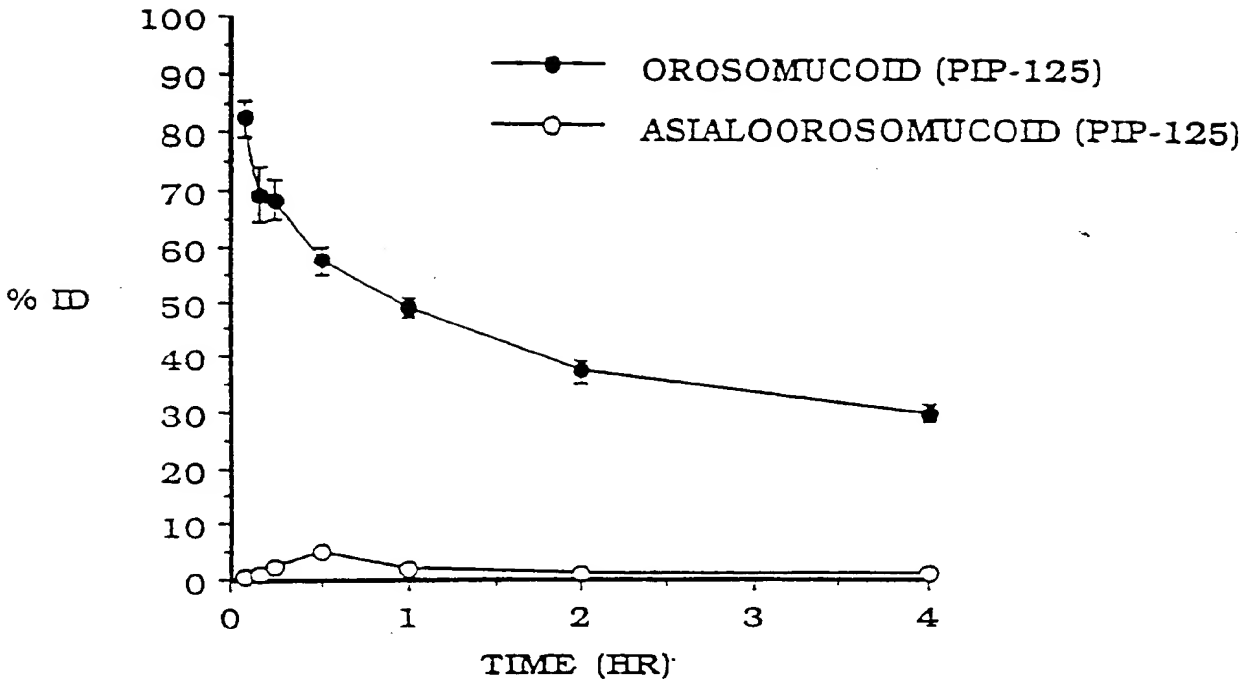

100
90
80
70
60
50
40
30
20
10
0
% ID
0
1
2
3
4
TIME (HR)
OROSOMUCOID (PIP-125)
ASIALOOROSOMUCOID (PIP-125)

Fig. 5.

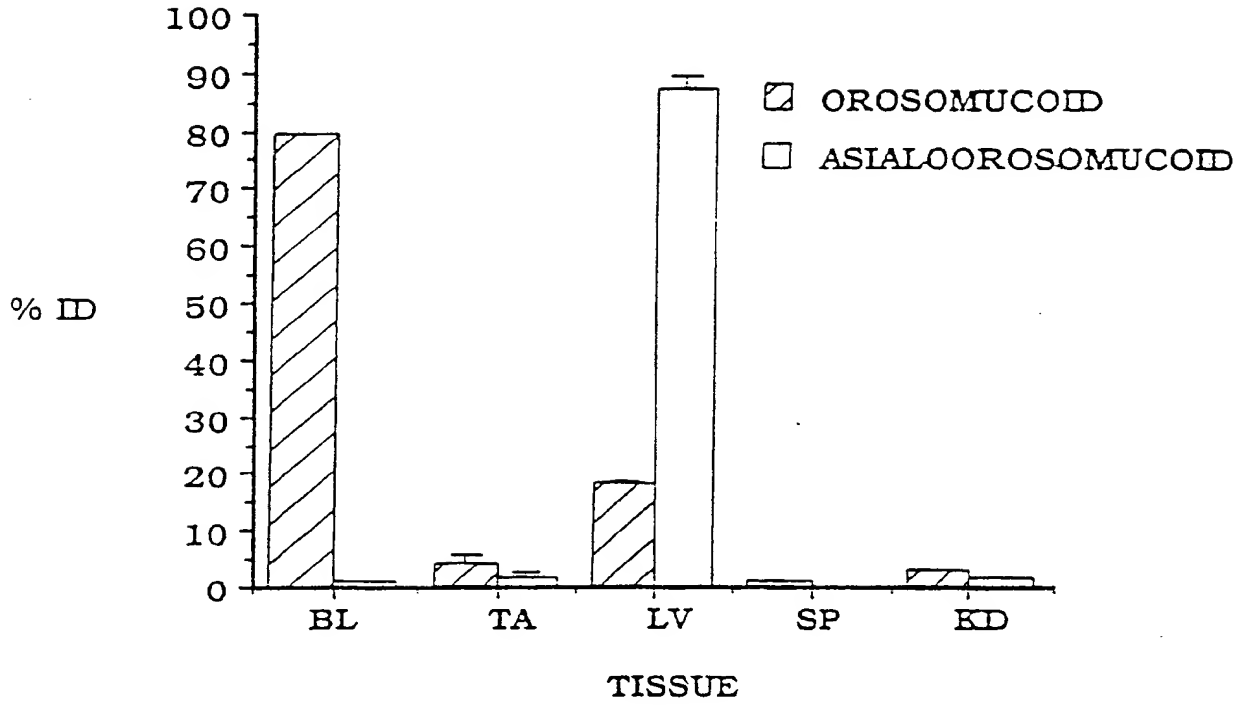

100
90
80
70
60
50
40
30
20
10
0
% ID
OROSOMUCOID
ASIALOOROSOMUCOID
BL
TA
LV
SP
KD
TISSUE

Fig. 6.

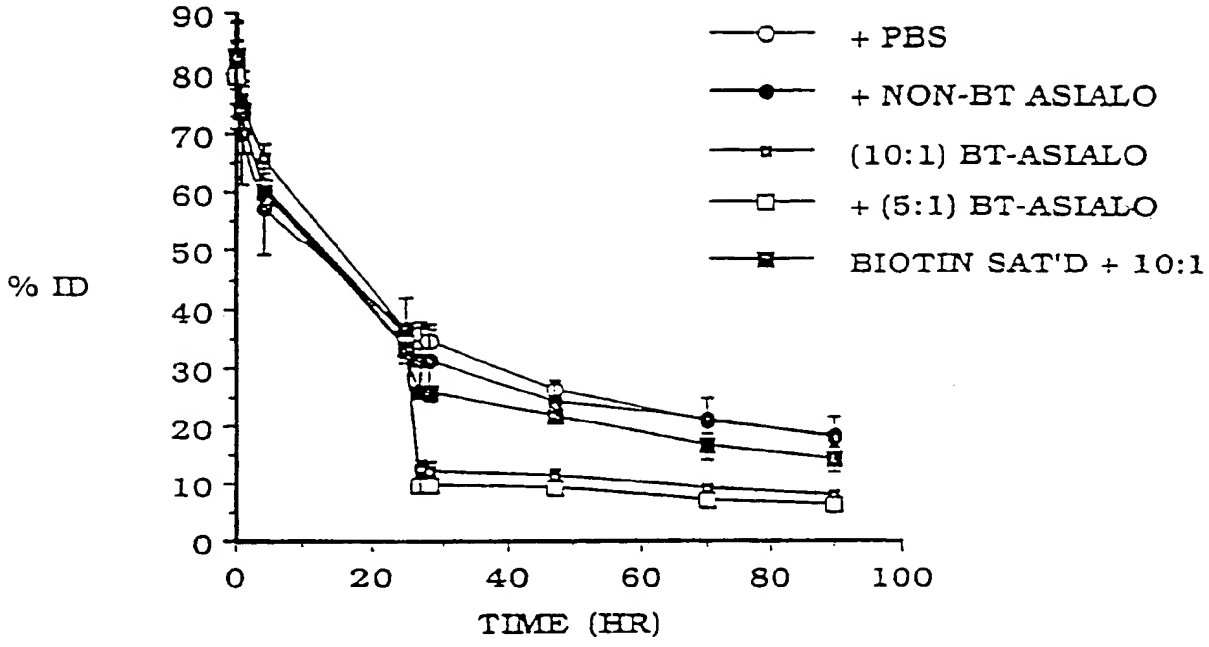

% ID
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
TIME (HR)
+ PBS
+ NON-BT ASIALO
(10:1) BT-ASIALO
+ (5:1) BT-ASIALO
BIOTIN SAT'D + 10:1

Fig. 7.

	%ID									
	+PBS	SD	+NON-BT	SD	+10:1	SD	+5:1	SD	BT-SAT'D	SD
BLOOD	31.05	5.08	29.94	1.35	8.54	0.91	7.03	0.18	24.58	0.68
TAIL	2.43	0.70	1.80	0.09	1.46	0.09	1.76	0.04	1.96	0.40
LUNG	1.47	0.26	1.09	0.22	0.54	0.10	0.48	0.07	0.76	0.01
LIVER	5.42	0.69	4.66	0.36	9.60	1.20	9.11	0.41	6.76	0.06
SPLEEN	0.25	0.05	0.34	0.03	0.17	0.03	0.18	0.00	0.38	0.02
STOMACH	0.28	0.02	0.33	0.03	0.53	0.34	0.49	0.00	0.29	0.04
KIDNEY	1.72	0.24	1.38	0.08	2.76	0.00	3.28	0.32	1.58	0.08
INTESTINE	3.40	0.73	3.44	0.10	4.22	0.02	6.62	0.14	2.83	0.13
	46.02		42.98		27.83		28.95		39.13	
	Group 1		Group 2		Group 3		Group 4		Group 5	

Fig. 8.

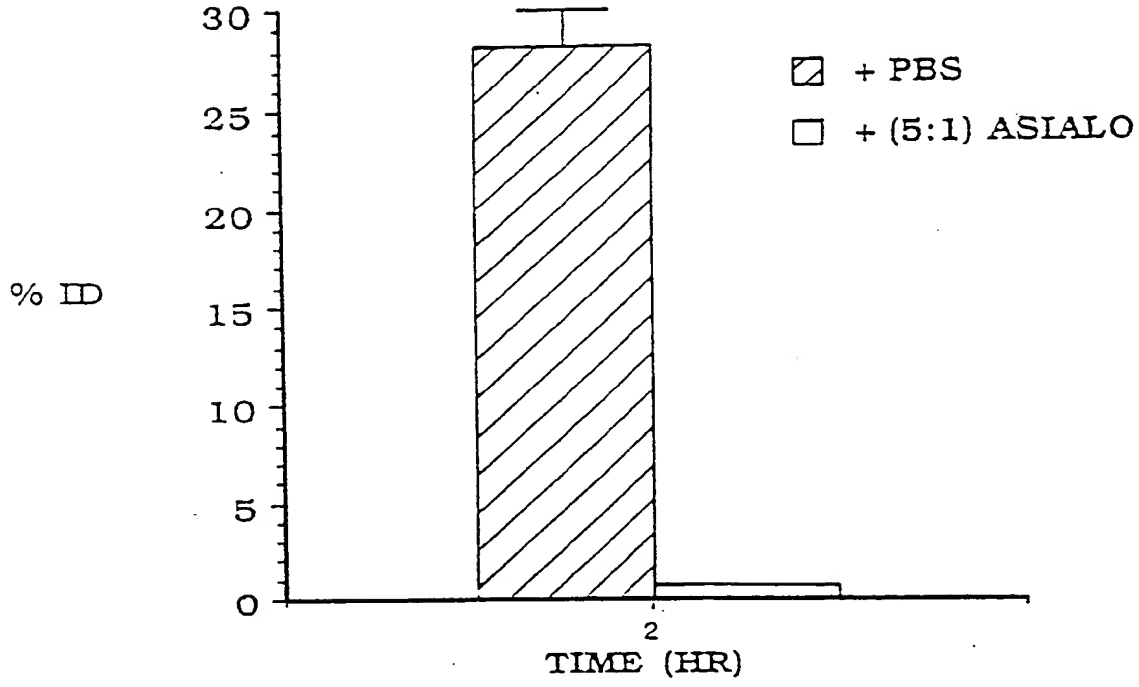


Fig. 9.

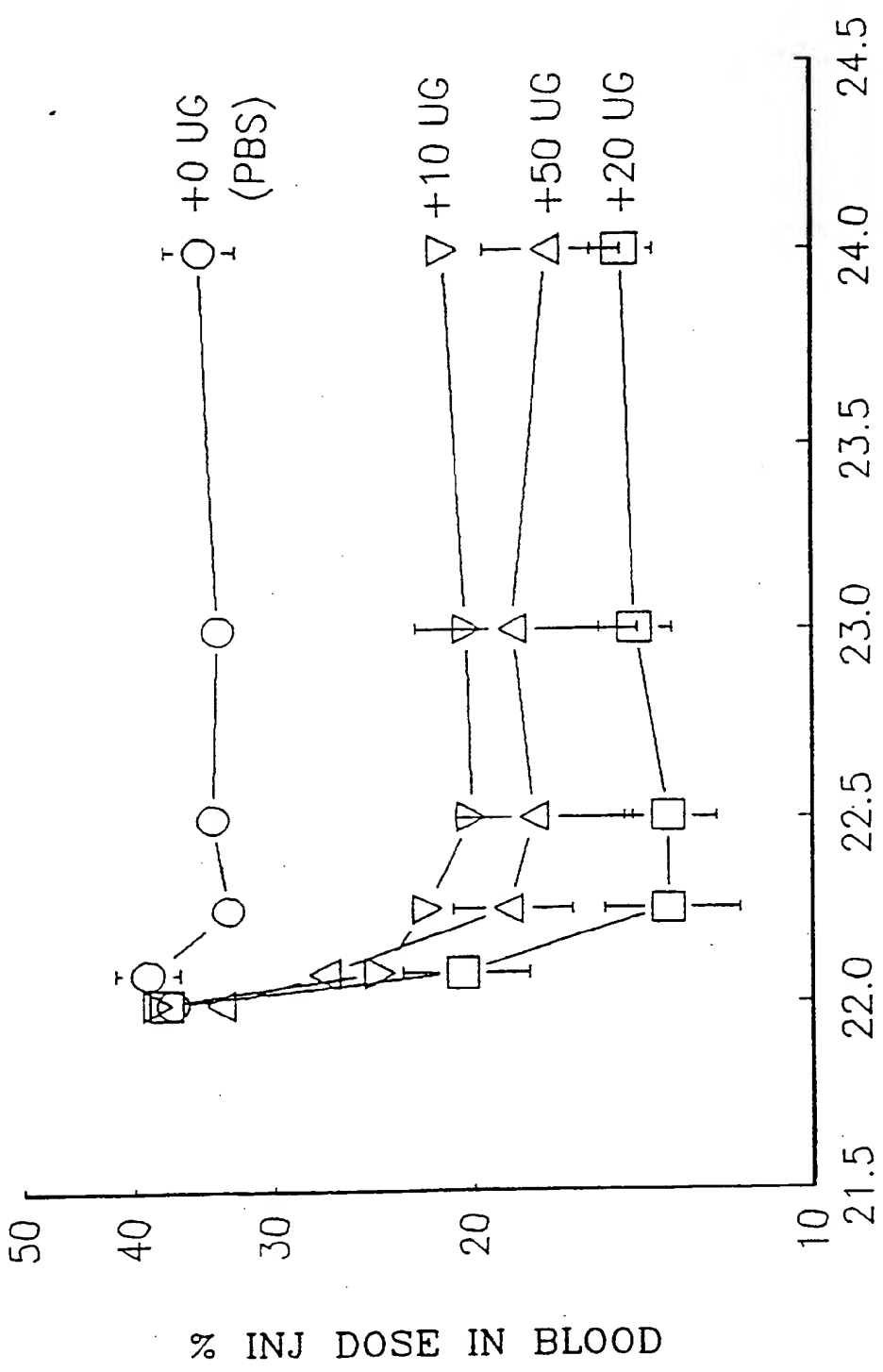

+0 UG
(PBS)
+10 UG
+50 UG
+20 UG
50
40
30
20
10
21.5
22.0
22.5
23.0
23.5
24.0
24.5
TIME (HR)
% INJ DOSE IN BLOOD

Fig. 10.

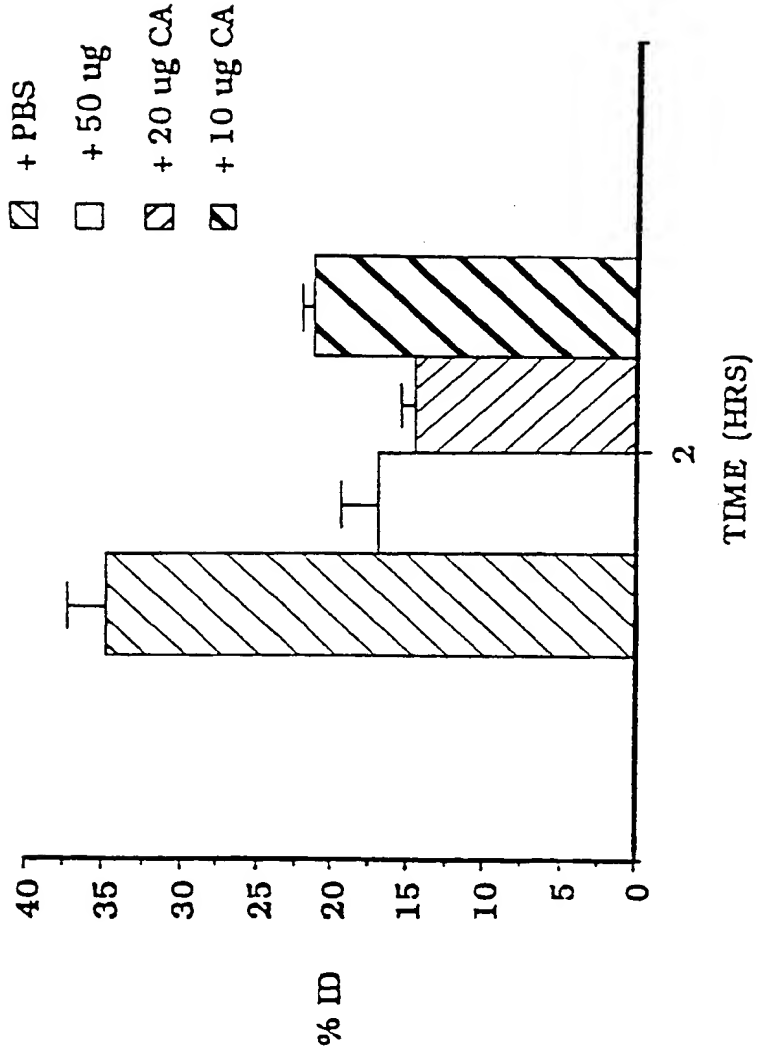


Fig. 11A.

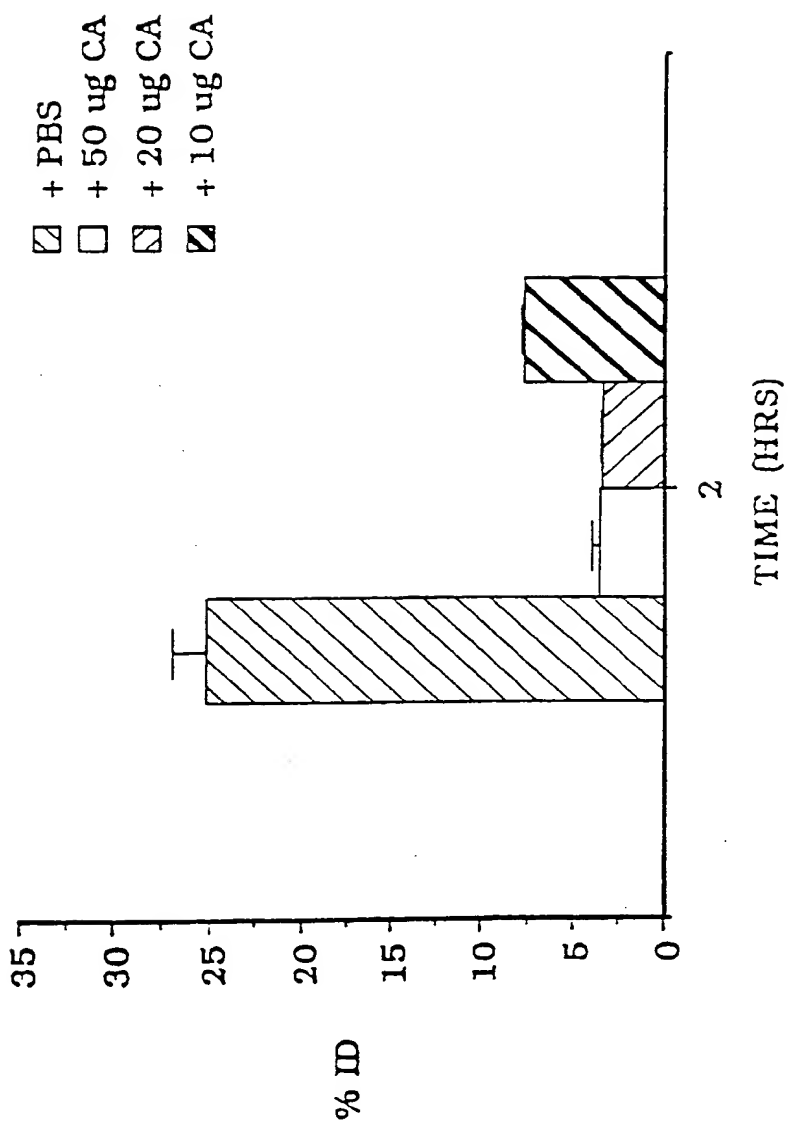


Fig. 11B.

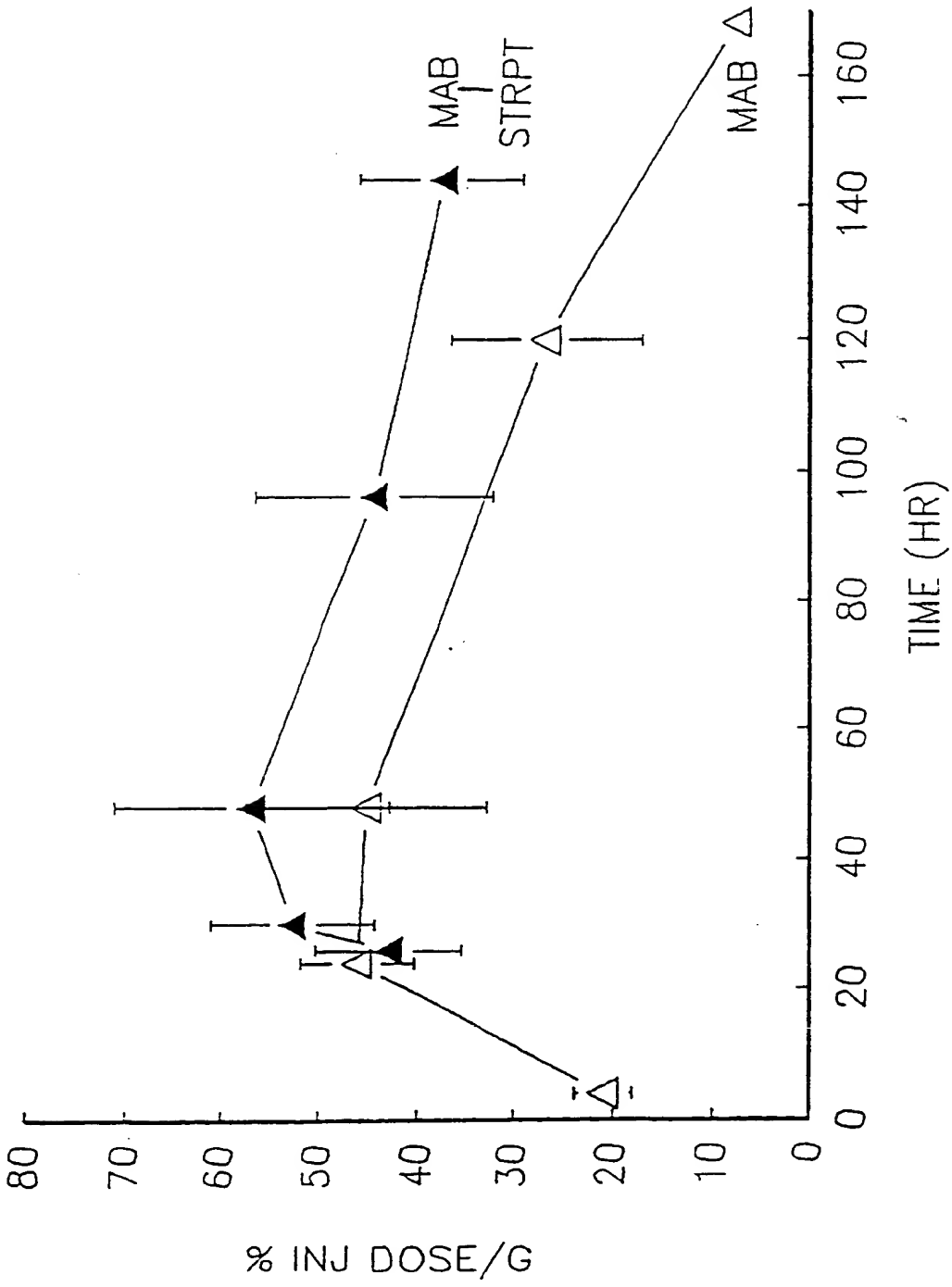

MAB
STRPT
MAB Δ
% INJ DOSE/G
TIME (HR)
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160

Fig. 12.

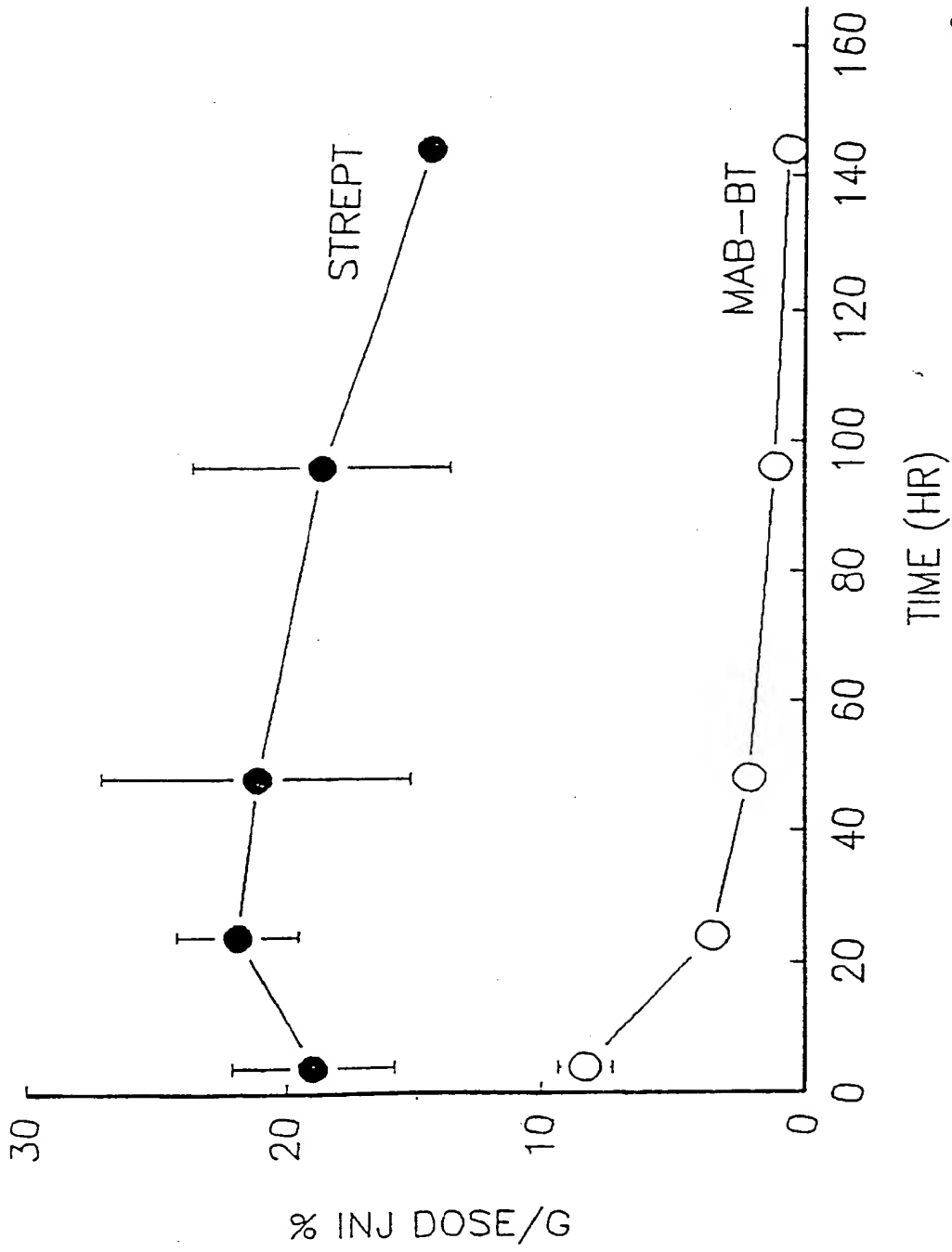

STREPT
MAB-BT
% INJ DOSE/G
TIME (HR)
0
10
20
30
0
20
40
60
80
100
120
140
160

Fig. 13.

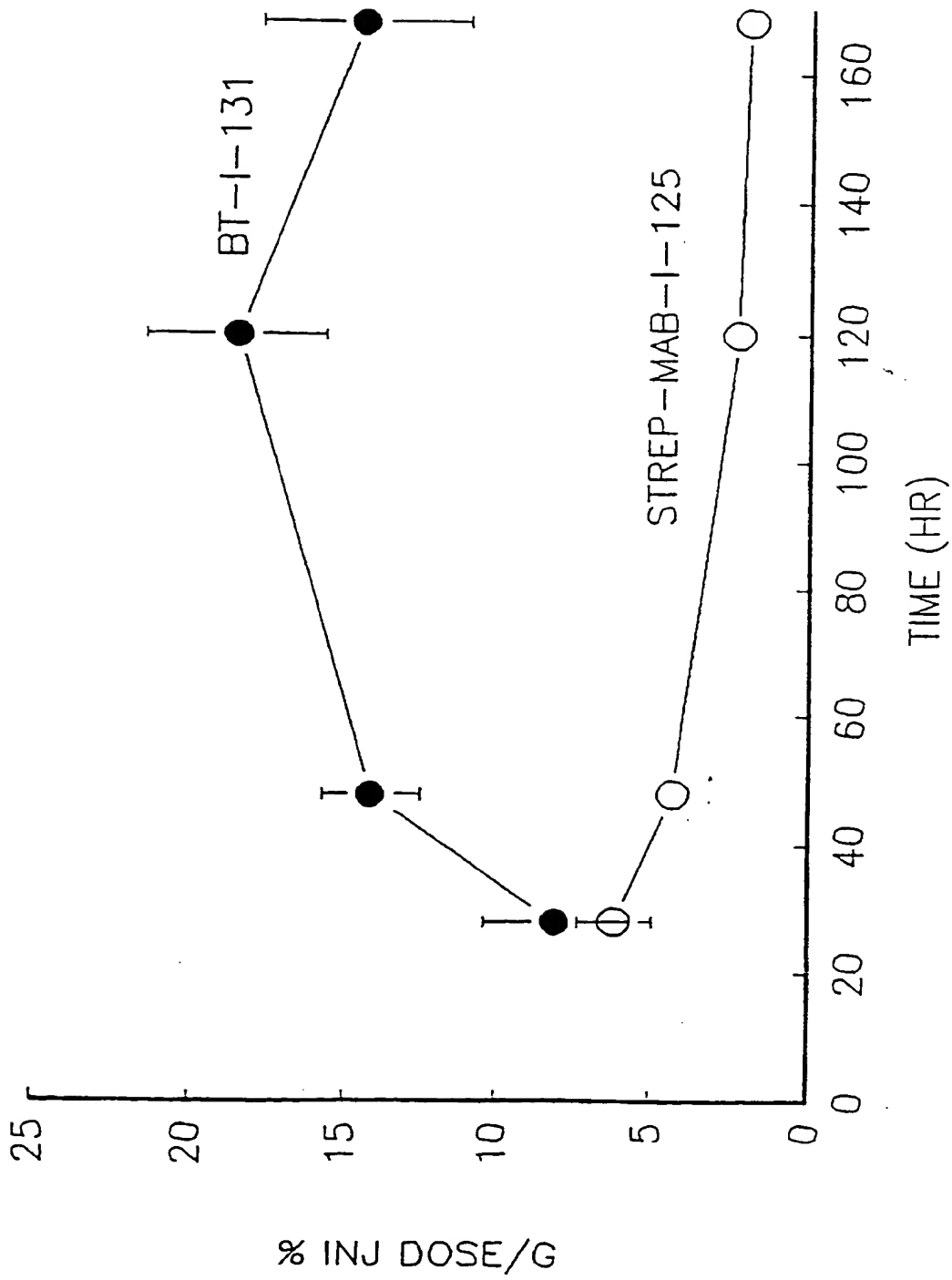

BT-I-131
STREP-MAB-I-125
% INJ DOSE/G
TIME (HR)
0
5
10
15
20
25
0
20
40
60
80
100
120
140
160

Fig. 14.

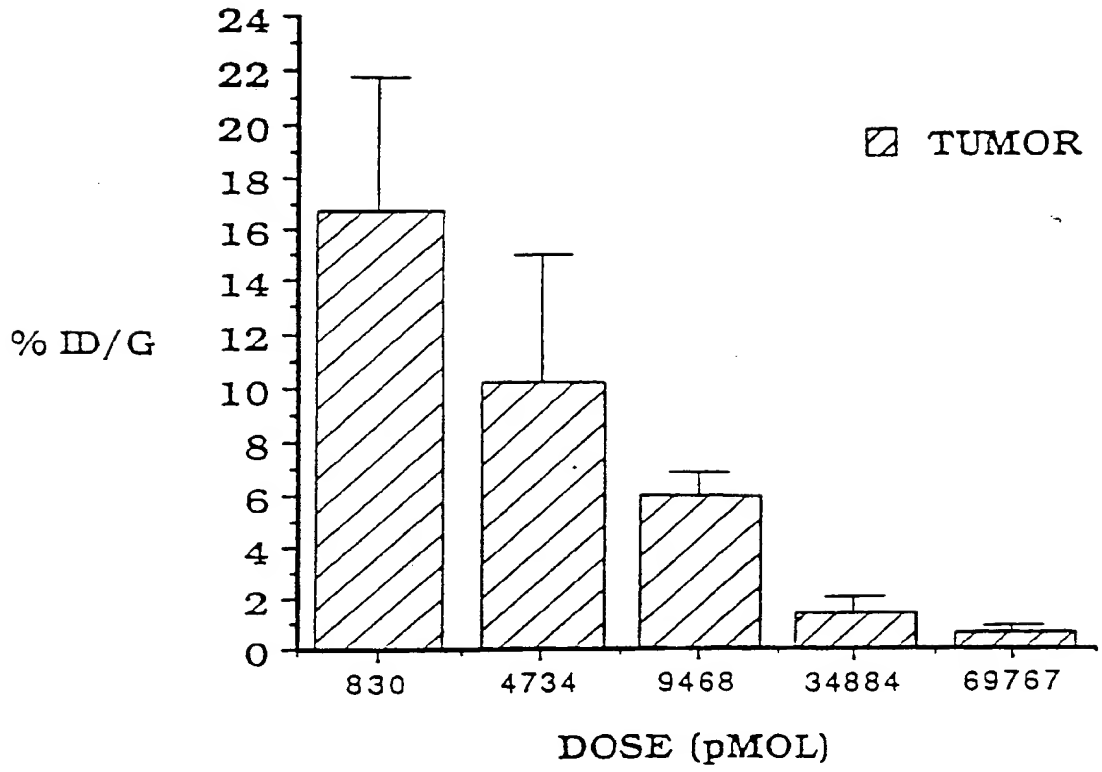

24
22
20
18
16
14
12
10
8
6
4
2
0
% ID/G
TUMOR
830
4734
9468
34884
69767
DOSE (pMOL)

Fig. 15A.

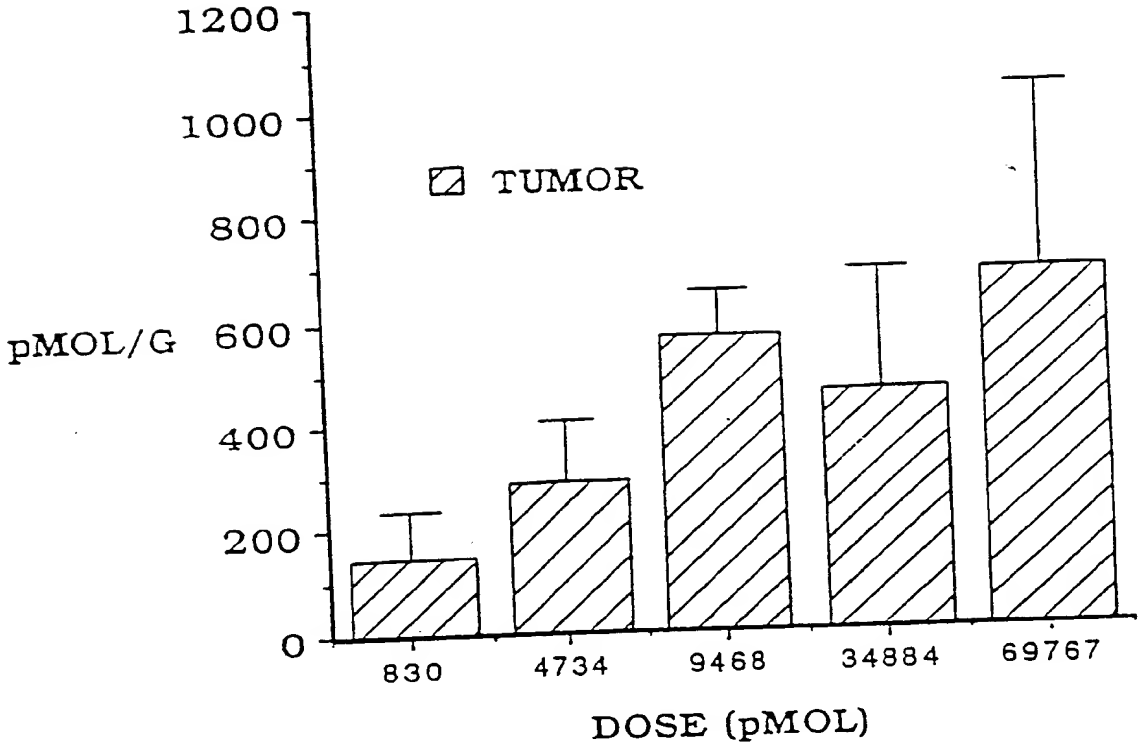

1200
1000
800
600
400
200
0
pMOL/G
TUMOR
830
4734
9468
34884
69767
DOSE (pMOL)

Fig. 15B.

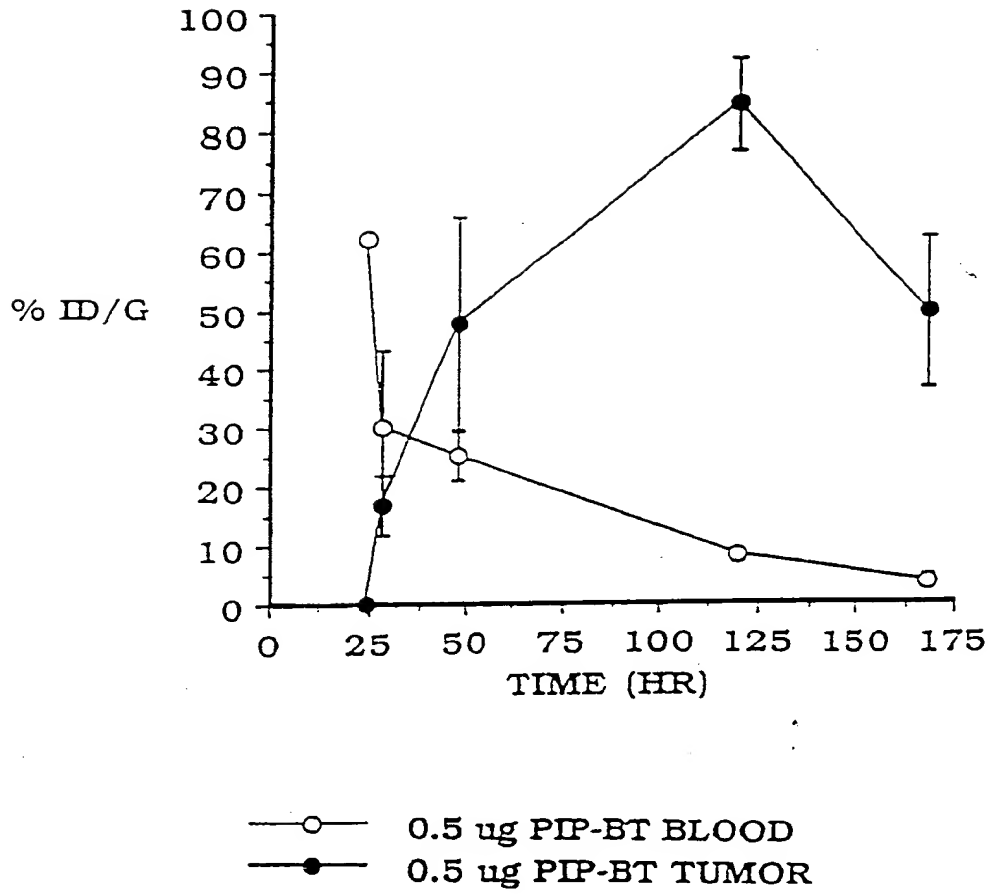

100
90
80
70
60
50
40
30
20
10
0
% ID/G
0
25
50
75
100
125
150
175
TIME (HR)
0.5 ug PIP-BT BLOOD
0.5 ug PIP-BT TUMOR

Fig. 16 A.

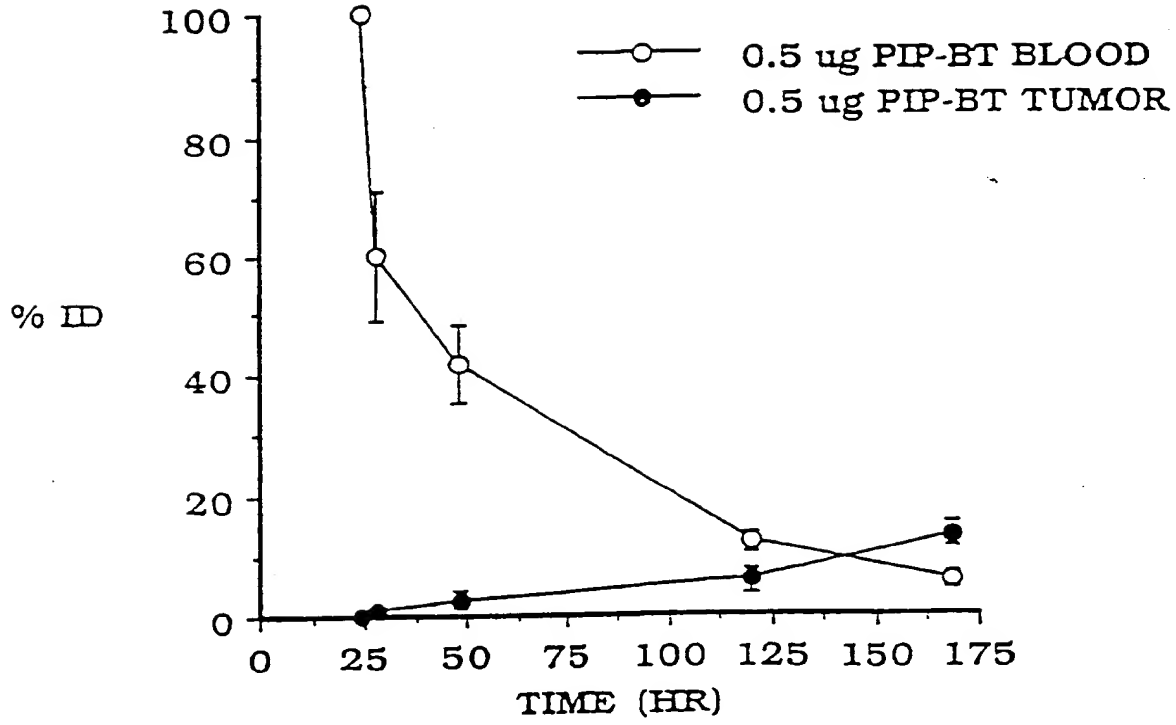

100
80
60
40
20
0
% ID
0
25
50
75
100
125
150
175
TIME (HR)
0.5 ug PIP-BT BLOOD
0.5 ug PIP-BT TUMOR

Fig. 16B.

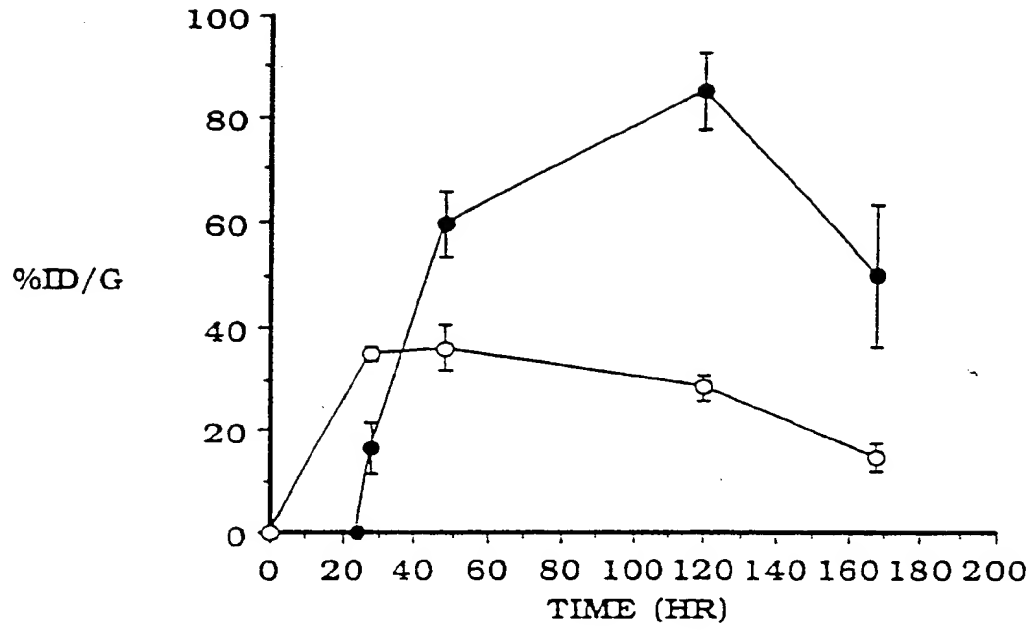

100
80
60
40
20
0
%ID/G
0 20 40 60 80 100 120 140 160 180 200
TIME (HR)
LU-10 StrAv TUMOR
PIP-BIOCYTIN TUMOR

Fig. 17A.

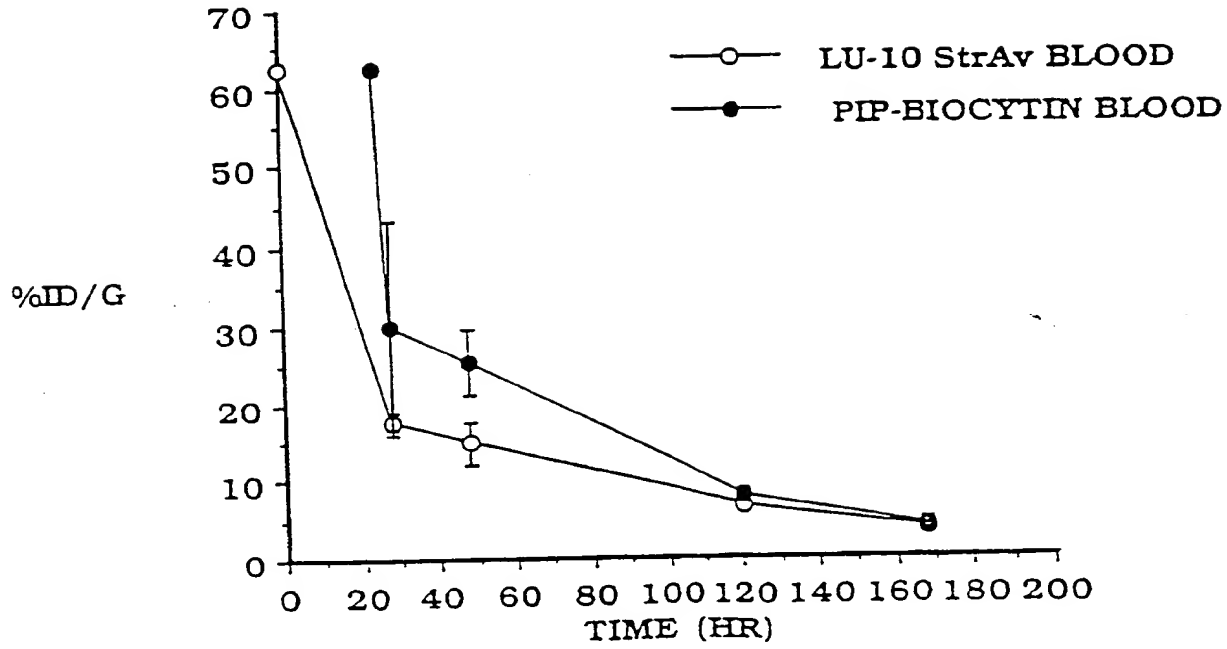

70
60
50
40
30
20
10
0
%ID/G
0 20 40 60 80 100 120 140 160 180 200
TIME (HR)
LU-10 StrAv BLOOD
PIP-BIOCYTIN BLOOD

Fig. 17B.

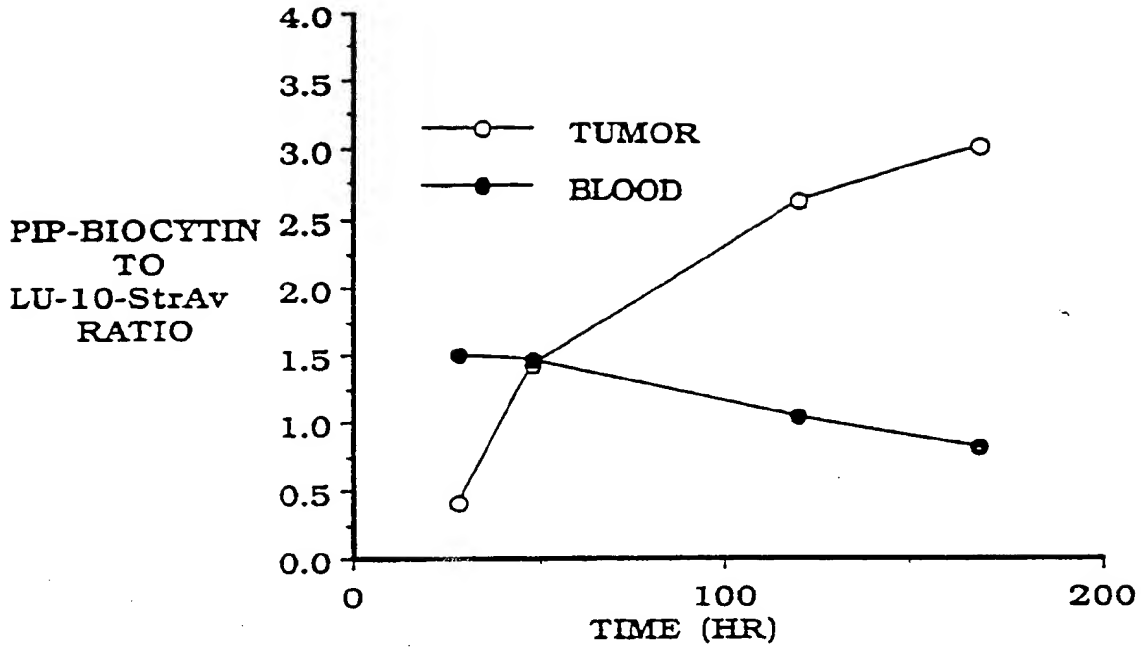

PIP-BIOCYTIN TO LU-10-StrAv RATIO
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TUMOR
BLOOD
0
100
200
TIME (HR)

Fig. 18.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14223

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 103:00, 103:10, 103:32, 51/00, 51/10, 49/00, 47/48

US CL :424/179.1, 181.1, 183.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/179.1, 181.1, 183.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/DIALOG, MEDLINE, BIOSIS, EMBASE, LIFESCI, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0,496,074 (PAGANELLI ET AL.) 29 JULY 1992, SEE ENTIRE DOCUMENT.	1-26
Y	NUCL. MED. COMM., VOLUME 12, ISSUED 1991, G. PAGANELLI ET AL., "MONOCLONAL ANTIBODY PRETARGETTING TECHNIQUES FOR TUMOUR LOCALIZATION: THE AVIDIN-BIOTIN SYSTEM", PAGES 211-233, SEE ENTIRE DOCUMENT.	1-26
Y	J. BIOL. CHEM., VOL. 252, NO. 11, ISSUED 10 JUNE 1977, A. ABUCHOWSKI ET AL., "EFFECT OF COVALENT ATTACHMENT OF POLYETHYLENE GLYCOL ON IMMUNOGENICITY AND CIRCULATING LIFE OF BOVINE LIVER CATALASE", PAGES 3582-3586 SEE ENTIRE DOCUMENT.	1-26
Y, P	US, A, 5,326,778 (ROSEBROUGH) 05 JULY 1994, SEE	1-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
28 FEBRUARY 1995	09 MAR 1995

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14223

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. PHARMACOL. EXP. THER., VOL 265, NO. 1, ISSUED APRIL 1993, S.F. ROSEBROUGH, "PLASMA STABILITY AND PHARMACOKINETICS OF RADIOLABELED DEFEROXAMINE-BIOTIN DERIVATIVES", PAGES 408-415, SEE ENTIRE DOCUMENT.	1-26
Y	HEPATOLOGY, VOL. 6, NO. 4, ISSUED 1986, P. VAN DER SLUIS ET AL., DRUG TARGETING TO THE LIVER WITH LACTOSYLATED ALBUMINS: DOES THE GLYCOPROTEIN TARGET THE DRUG OR IS THE DRUG TARGETING THE GLYCOPROTEIN?", PAGES 723-728, SEE ENTIRE DOCUMENT.	1-26
Y	J. NUCL. MED., VOL. 33, 5TH SUPPL., ISSUED 13 MAY 1992, J.A. SANDERSON ET AL., "PREPARATION AND CHARACTERIZATION OF BIOTIN CONJUGATES OF ANTI-PAN-CARCINOMA NR-LU-10 MONOCLONAL ANTIBODY FOR A THREE STEP RADIOIMMUNOTHERAPY", ABSTRACT 233, PAGE 880, SEE ENTIRE DOCUMENT.	1-26
Y	BR. J. CANCER, VOL. 58, VOL. 5, ISSUED NOVEMBER 1988, J.A. LEDERMAN ET AL., "REPEATED ANTITUMOUR ANTIBODY THERAPY IN MAN WITH SUPRESSION OF THE HOST RESPONSE BY CYCLOSPORIN A", PAGES 654-657, SEE ENTIRE DOCUMENT.	1-26